Award Number:
W81 XWH-10-1-0740

TITLE:
Maintenance of Epithelial Stem Cells by Cbl Proteins

PRINCIPAL INVESTIGATOR:
Mayumi Naramura, M.D.

CONTRACTING ORGANIZATION:
University of Nebraska Medical Center
Omaha, Nebraska 68198

REPORT DATE:
September 2012

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September2012	Annual	1 Sept 2011 - 31 Aug 2012

4. TITLE AND SUBTITLE

Maintenance of Epithelial Stem Cells by Cbl Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0740

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mayumi Naramura, M.D.

E-Mail: mnaramura@unmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Nebraska Medical Center

Omaha, Nebraska 68198

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Cbl family E3 ubiquitin ligases are critical regulators of tyrosine kinase-mediated signal transduction, and we and others demonstrated that the Cbl family proteins were required to maintain the homeostasis of the hematopoietic stem cell compartment and disturbance of this critical regulatory pathway led to cancer in human and mice. However, their roles in the epithelial stem/progenitor cells has never been examined. Using the newly-established hTERT-immortalized human mammary epithelial cell (hMEC) line (hereafter called TERT-hMEC), we demonstrated that loss of Cbl inhibited differentiation of epithelial cells. Our preliminary studies indicate that Cbl functions in multiple signaling pathways beyond the traditional EGFR pathway, which appears to be responsible for the complex phenotypes of Cbl deficiency in the epithelial compartment.

15. SUBJECT TERMS
breast cancer, mammary epithelial stem cells Cbl, ubiquitin ligase

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	78	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION

Advances in molecular typing identified several subtypes of breast cancer which show distinct prognosis and responses to treatment, and the prevailing concept has been that these different subtypes originate from breast epithelial cells at distinct developmental stages, although recent findings begin to challenge this conjecture [1] [2] [3]. Nevertheless, it is imperative to better understand the molecular mechanisms of normal mammary gland development and apply this knowledge to pathophysiology of breast cancer. The Cbl family E3 ubiquitin ligases are critical regulators of tyrosine kinase-mediated signal transduction [4], and we and others demonstrated that the Cbl family proteins were required to maintain the homeostasis of the hematopoietic stem cell compartment [5] [6] [7]. However, the role of the Cbl family proteins in the epithelial stem/progenitor cells has never been examined. Therefore, we formed a hypothesis that the Cbl family proteins are required for the maintenance of stem/progenitor homeostasis in the mammary epithelium, and that loss of this regulatory control will enlarge the stem/progenitor pool and predispose the mammary gland to oncogenesis. To test this hypothesis, we proposed to employ two complementary approaches, one involving the use of a newly-established hTERT-immortalized human mammary epithelial cell (hMEC) line (hereafter called TERT-hMEC) [8], and the gene knock-out mouse strains.

BODY

In order to test the hypothesis above, we proposed following Specific Aims:

Aim 1. Define the role of the Cbl family protein in progenitor-type mammary epithelial cells in culture

Aim 2. Define the role of the Cbl family proteins in mammary gland development and tumorigenesis in vivo.

and following specific tasks were identified:

Task 1: Define the role of the Cbl family proteins in progenitor-type mammary epithelial cells in culture (Months 1-18)

1-a. Establish the base line for flow cytometry markers and flow-based functional analyses (Hoechst dye exclusion, ALDEFLUOR assay) (Months 1-6)

1-b. Establish mammosphere culture and differentiation assay conditions for progenitor-type MEC lines (Months 4-9)

1-c. Generate Cbl/Cbl-b double deficient human progenitor MEC lines using shRNA (Months 7-12)

1-d. Characterize control and Cbl/Cbl-b double deficient MECs by flow cytometry and functional analyses (Months 13-24)

Task 2: Define the biochemical pathways affected by Cbl/Cbl-b loss by microarray analyses (Months 13-24)

2-a. Perform microarray analyses on control and Cbl/Cbl-b double deficient MECs growing in nondifferentiating conditions (Months 13-18)

2-b. Perform microarray analyses on control and Cbl/Cbl-b double deficient MECs undergoing lineage differentiation (Months 19-24)

Task 3: Define the role of the Cbl family proteins in mammary gland development and tumorigenesis in vivo (Months 1-24)

3-a. Generate sufficient number of MMTV-Cre;Cbl(flox/flox);Cblb(-/-) for mammary gland transplant (Months 1-12)

3-b. Characterize transplanted mammary gland phenotypes by histology, immunohistochemistry and flow cytometry (Months 7-18)
3-c. Characterize transplanted mammary gland phenotypes by serial transplantation and tissue regeneration assay (Months 13-24)

In the previous report covering Sept. 2010 - Aug. 2011, we described the completion of following tasks:

- Established the in vitro cell differentiation assay of the TERT-hMECs.
- Generated inducible shRNA-mediated Cbl knock-down in the TERT-hMECs.
- Demonstrated that acute Cbl knock-down inhibits TERT-hMEC differentiation in vitro.
- Demonstrated that the mammary gland development is altered in MMTV-Cre;Cbl(flox/flox);Cblb(-/-) mice.

During the present reporting period (Sept. 2011 - Aug. 2012), we investigated the mechanisms of MEC differentiation and the roles of Cbl family proteins in this process using the reagents generated during the previous reporting period.

We first confirmed that acute Cbl knock-down inhibits in vitro differentiation of the TERT-hMECs. Previously, we defined cell differentiation based on morphology and immunofluorescence microscopy. While useful, these assays can be subjective, nor ideally suited for quantitative analyses. Therefore, we established a flow cytometry-based assay to define MEC differentiation. As shown in Figure 1, undifferentiated TERT-hMECs express EpCAM and CD49f on the cell surface. When cultured under differentiation-promoting conditions, two novel cell populations emerged, one characterized by the reduction of the surface EpCAM (EpCAM neg/lo) and the other with the high levels of EpCAM but the reduced expression of CD49f (EpCAM hi/CD49f int). Further studies revealed that the EpCAM neg/lo cells lost the expression of Cytokeratin 5 (K5) and elevated smooth muscle actin (SMA), consistent with myoepithelial differentiation. On the other hand, EpCAM hi/CD49f int cells were negative for SMA, but stained positive for MUC1, consistent with luminal differentiation. Using this system, we re-evaluated the effect of acute Cbl knock-down with the inducible shRNA. As shown in Figure 2, we confirmed by flow cytometry that acute Cbl knock-down interfered with the TERT-hMEC differentiation as we previously reported based on morphology and immunofluorescence.

We and others previously reported that Erk activity was upregulated in the absence of Cbl in various systems. Thus, we examined Erk activities in TERT-hMECs upon Cbl knock-down. As shown in Figure 3, Cbl-deficient MECs showed sustained Erk activation when stimulated with EGF, as expected. These results are, however, inconsistent with a recent report by Pasic and colleagues which demonstrated that sustained activation of Erk promoted myoepithelial differentiation of primary human MECs. Why did Cbl knock-down TERT-hMECs fail to differentiate in spite of sustained Erk activation? It is conceivable that this is due to intrinsic differences between the primary MECs used by Pasic et al. and the hTERT-immortalized MECs in our system. To address this, we substituted EGF in the differentiation-inducing culture media with other EGF receptor ligands which are known to induce varying degrees of Erk activation, and compared their effects. As shown in Figure 4, when EGF was substituted with amphiregulin (Areg), TERT-hMECs only differentiated into luminal cells whereas TGF alpha, another EGFR ligand supported only myoepithelial differentiation. These results were confirmed by

morphology, immunofluorescence and flow cytometry. Consistent with previous reports [9], engagement of the EGFR with Areg led to a transient phosphorylation of Erk whereas the stimulation with TGF alpha resulted in a sustained activation of this pathway (Figure 5). Thus, we conclude that the TERT-hMEC system is capable to recapitulate the Erk-dependent MEC differentiation reported in primary human MEC.

Then, why does the Erk activation induced by the loss of Cbl lead to impaired differentiation of MECs? As an initial effort to address this question, we probed Cbl-deficient TERT-hMECs with antibodies against Akt and found that Akt phosphorylation was enhanced (Figure 6). This is consistent with previously-reported roles of Cbl as a negative regulator of the PI3 kinase-Akt pathway [10]. Among various downstream effectors of the Akt pathway, GSK-3 beta is a ubiquitously expressed serine/threonine protein kinase whose activity can be inhibited by Akt-mediated phosphorylation at Ser9. Active GSK3 phosphorylates beta catenin and promotes its degradation [11]. Thus, inhibition of GSK3 activity, i.e., increased phosphorylation at Ser9, is linked to stabilization of beta catenin. While phosphorylation of beta catenin by GSK3 is a signal for destabilization, phosphorylation of this protein at Ser552 is known to induce protein accumulation, nuclear transport and subsequent elevation of the transcriptional activity. As shown in Figure 6, phosphorylation of GSK3 beta at Ser9 (inactivating phosphorylation) and that of beta catenin at Ser552 (activating phosphorylation) was enhanced in Cbl knock-down TERT-hMECs. Because enhanced beta catenin activity is associated with stem cell maintenance in various systems, we consider that the activation of the beta catenin pathway may be a potential mechanism behind inhibition of MEC differentiation in the absence of Cbl.

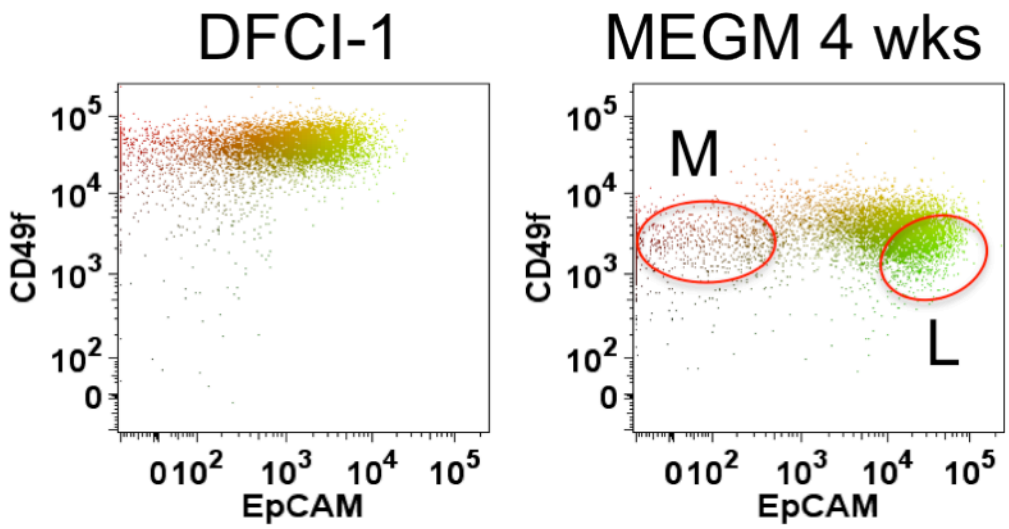


Figure 1. Flow cytometry analysis of hTERT-immortalized hMEC differentiation. Cells maintained under non-differentiating (DFCI-1 medium, left) or differentiating (MEGM medium, right) conditions were stained with antibodies against EpCAM and CD49f and analyzed by flow cytometry. L, luminal population; M, myoepithelial population.

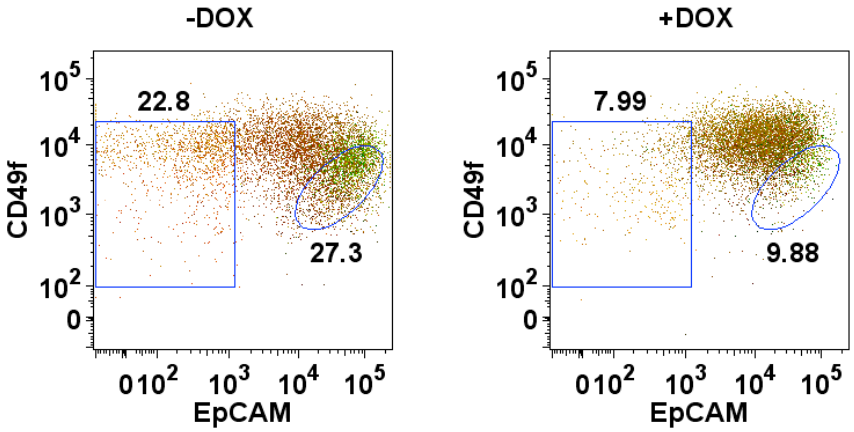


Figure 2. Effects of acute Cbl knock-down on TERT-hMEC differentiation. In the absence of doxycycline, i.e., with normal Cbl expression (-DOX, left), 27.3 % of the cells differentiated into luminal and 22.8 % of the cells differentiated into myoepithelial cells. However, when Cbl shRNA was expressed by doxycycline (+DOX, right), cell differentiation was significantly inhibited.

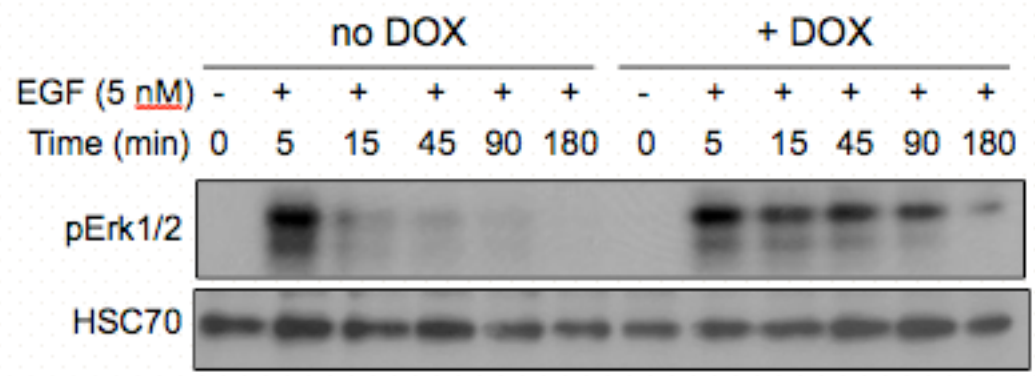


Figure 3. Cbl knock-down prolongs Erk activation. TERT-hMECs were either pre-treated with doxycycline to induce Cbl knock-down or left untreated, starved of EGF for 48 hours, stimulated with EGF and cell lysates were analyzed by immunoblotting for phosphorylated Erk.

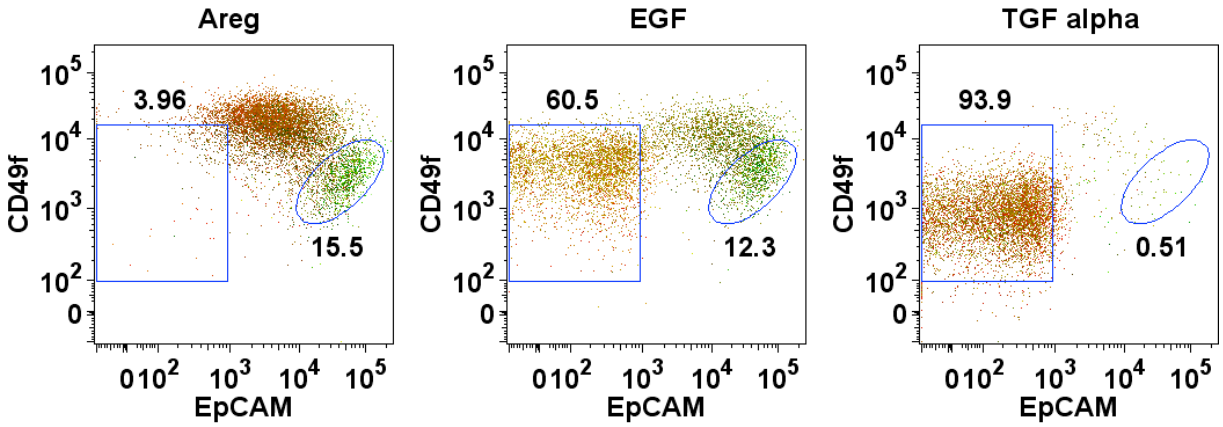


Figure 4. Effects of various EGF receptor ligands on the TERT-hMEC differentiation. EGF was substituted with various EGF receptor ligands, i.e., amphiregulin (Areg) or TGF alpha in the differentiation-promoting MEGM.

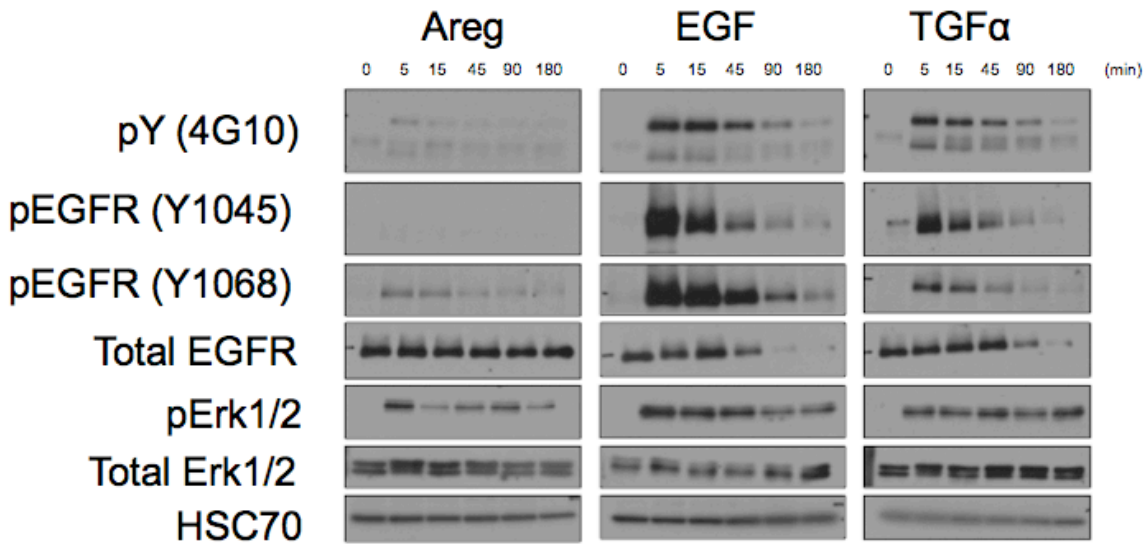


Figure 5. Biochemical consequences of EGF receptor engagement with various ligands. TERT-hMECs were starved of EGF for 48 hours prior to stimulation with indicated ligands. Cell lysates were analyzed by immunoblotting.

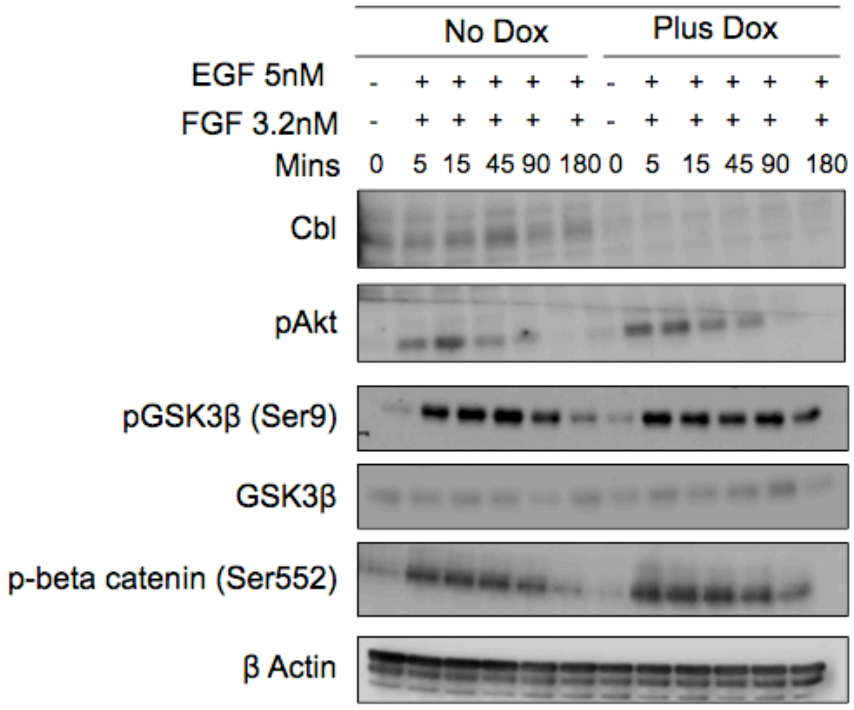


Figure 6. Cbl knock-down enhances beta catenin activation. TERT-hMECs were either pre-treated with doxycycline to induce Cbl knock-down or left untreated, starved of EGF for 48 hours, stimulated with EGF and FGF and cell lysates were analyzed by immunoblotting.

KEY RESEARCH ACCOMPLISHMENTS

- Established flow cytometry-based mammary epithelial cell (MEC) differentiation analysis platform
- Investigated the effects of various EGF receptor ligands on the MEC differentiation and demonstrated that the in vitro TERT-hMEC line model is well suited to recapitulate previously-published results on primary human mammary epithelial cells
- Obtained preliminary data indicating that, while enhanced Erk activation predisposes MECs to differentiate into myoepithelial cells when this activation is delivered through EGF receptor engagement, additional pathways are likely to participate in the ultimate cell fate decision.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Review article on Cbl:

- Nadeau S, An W, Palermo N, Feng D, Ahmad G, Dong L, Borgstahl GEO, Natarajan A, **Naramura M,** Band V, Band H. Oncogenic signaling by leukemia-associated mutant Cbl proteins. Submitted.

- Mohapatra B, Band H, Ahmad G, Nadeau S, Zutshi N, An W, Scheffee S, Dong L, Feng D, Goetz B, Borgstahl G, Natarajan A, Raja SK, **Naramura M**, Band V. Protein tyrosine kinase regulation by ubiquitination: critical roles of Cbl family ubiquitin ligases. Submitted.

Mammary epithelial cell biology:

- Helikar T, Kochi N, Kowal B, Dimri M, **Naramura M,** Raja SM, Band V, Band H, Rogers J. Src induces EGFR internalization under EGF-deprived conditions: a systems approach. Submitted.

- Ortega-Cava CF, Raja SM, Laiq Z, Bailey TA, Luan H, Mohapatra B, Williams SH, Ericsson AC, Goswami R, Dimri M, Duan L, Band V, **Naramura M**, Band H. Continuous requirement of ErbB2 kinase activity for loss of cell polarity and lumen formation in a novel ErbB2/Neu-driven murine cell line model of metastatic breast cancer. J Carcinog. 2011;10:29. Epub 2011 Nov 30. PubMed PMID: 22190871; PubMed Central PMCID: PMC3243085.

- Bailey TA, Luan H, Clubb RJ, **Naramura M**, Band V, Raja SM, Band H. Mechanisms of Trastuzumab resistance in ErbB2-driven breast cancer and newer opportunities to overcome therapy resistance. J Carcinog. 2011;10:28. Epub 2011 Nov 30. PubMed PMID: 22190870; PubMed Central PMCID: PMC3243087.

Other research articles published during this grant period:

- Mohibi S, Gurumurthy CB, Nag A, Wang J, Mirza S, Mian Y, Quinn M, Katafiasz B, Eudy J, Pandey S, Guda C, **Naramura M**, Band H, Band V. Mammalian alteration/deficiency in activation 3 (ada3) is essential for

embryonic development and cell cycle progression. J Biol Chem. 2012 Aug 24;287(35):29442-56. Epub 2012 Jun 26. PubMed PMID: 22736770; PubMed Central PMCID: PMC3436190.

- Tu C, Ahmad G, Mohapatra B, Bhattacharyya S, Ortega-Cava CF, Chung BM, Wagner KU, Raja SM, **Naramura M**, Band V, Band H. ESCRT proteins: Double-edged regulators of cellular signaling. Bioarchitecture. 2011 Jan;1(1):45-48. PubMed PMID: 21866262; PubMed Central PMCID: PMC3158637.

Presentations

- 2012 Midwest Student Biomedical Research Forum (Omaha, NE)
- 2012 Gordon Research Conference on Mammary Gland Biology (Lucca (Barga), Italy)
- 2012 Cold Spring Harbor Laboratory Meeting on Mechanisms and Models of Cancer (Cold Spring Harbor, NY)

Funding

- Skala Fellowship awarded to Chandrani Mukhopadhyay (graduate student in the lab)

CONCLUSION

In the second year of this DOD-supported project, we primarily focused on the molecular/biochemical mechanisms of MEC differentiation. As shown here, sustained activation of the Erk pathway initiated by the EGF receptor engagement promotes MEC differentiation towards the myoepithelial lineage; these results are consistent with previous reports in primary human MECs and provide strong evidence that this cell line is a tractable model for normal human MEC differentiation. Loss of Cbl in the TERT-hMEC interfered with differentiation. Unlike hematopoietic system, loss of Cbl proteins in the epithelial compartment did not lead to cell expansion. A separate line of our own evidence (performed outside of the current DOD-supported project) suggests that Cbl loss in the mammary gland does not predispose mice to mammary tumors. Taken together, these results are indicative of the presence of distinct mechanisms regulating the homeostasis of hematopoietic and epithelial systems which remain to be clarified.

REFERENCES

1. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U.S.A. 2001; 98: 10869-10874.
2. Visvader JE. Cells of origin in cancer. Nature. 2011; 469: 314-322.
3. Molyneux G, Smalley MJ. The cell of origin of BRCA1 mutation-associated breast cancer: a cautionary tale of gene expression profiling. J Mammary Gland Biol Neoplasia. 2011; 16:51-5.

4. Duan L, Reddi AL, Ghosh A, Dimri M, Band H. The Cbl family and other ubiquitin ligases: destructive forces in control of antigen receptor signaling. Immunity. 2004; 21: 7-17.
5. Rathinam C, Thien CBF, Langdon WY, Gu H, Flavell RA. The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells. Genes Dev. 2008; 22: 992-997.
6. Naramura M, Nandwani N, Gu H, Band V, Band H. Rapidly fatal myeloproliferative disorders in mice with deletion of Casitas B-cell lymphoma (Cbl) and Cbl-b in hematopoietic stem cells. Proc. Natl. Acad. Sci. U.S.A. 2010; 107: 16274-16279.
7. Rathinam C, Thien CBF, Flavell RA, Langdon WY. Myeloid leukemia development in c-Cbl RING finger mutant mice is dependent on FLT3 signaling. Cancer Cell. 2010; 18: 341-352.
8. Zhao X, Malhotra GK, Lele SM, Lele MS, West WW, Eudy JD, Band H, Band V. Telomerase-immortalized human mammary stem/progenitor cells with ability to self-renew and differentiate. Proc. Natl. Acad. Sci. U.S.A. 2010; 107: 14146-14151.
9. Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, Grøvdal L, Willumsen BM, van Deurs B. Differential effects of EGFR ligands on endocytic sorting of the receptor. Traffic. 2009; 10: 1115-27.
10. Rathinam C, Thien CB, Flavell RA, Langdon WY. Myeloid leukemia development in c-Cbl RING finger mutant mice is dependent on FLT3 signaling. Cancer Cell. 2010; 18: 341-52.
11. Alexander CM, Goel S, Fakhraldeen SA, Kim S. Wnt signaling in mammary gland: plastic cell fates and combinatorial signaling. Cold Spring Harb Perspect Biol. published online June 1, 2012.

APPENDICES

- Ortega-Cava CF, Raja SM, Laiq Z, Bailey TA, Luan H, Mohapatra B, Williams SH, Ericsson AC, Goswami R, Dimri M, Duan L, Band V, Naramura M, Band H. Continuous requirement of ErbB2 kinase activity for loss of cell polarity and lumen formation in a novel ErbB2/Neu-driven murine cell line model of metastatic breast cancer. J Carcinog. 2011;10:29. Epub 2011 Nov 30. PubMed PMID: 22190871; PubMed Central PMCID: PMC3243085.

- Bailey TA, Luan H, Clubb RJ, Naramura M, Band V, Raja SM, Band H. Mechanisms of Trastuzumab resistance in ErbB2-driven breast cancer and newer opportunities to overcome therapy resistance. J Carcinog. 2011;10:28. Epub 2011 Nov 30. PubMed PMID: 22190870; PubMed Central PMCID: PMC3243087.

- Mohibi S, Gurumurthy CB, Nag A, Wang J, Mirza S, Mian Y, Quinn M, Katafiasz B, Eudy J, Pandey S, Guda C, Naramura M, Band H, Band V. Mammalian alteration/deficiency in activation 3 (ada3) is essential for embryonic development and cell cycle progression. J Biol Chem. 2012 Aug 24;287(35):29442-56. Epub 2012 Jun 26. PubMed PMID: 22736770; PubMed Central PMCID: PMC3436190

- Tu C, Ahmad G, Mohapatra B, Bhattacharyya S, Ortega-Cava CF, Chung BM, Wagner KU, Raja SM, Naramura M, Band V, Band H. ESCRT proteins: Double-edged regulators of cellular signaling. Bioarchitecture. 2011 Jan;1(1):45-48. PubMed PMID: 21866262; PubMed Central PMCID: PMC3158637.

Journal of Carcinogenesis

Home
Browse articles
Instructions
Submit articles

J Carcinog. 2011; 10: 29. PMCID: PMC3243085
Published online 2011 November 30. doi: 10.4103/1477-3163.90443

Continuous requirement of ErbB2 kinase activity for loss of cell polarity and lumen formation in a novel ErbB2/Neu-driven murine cell line model of metastatic breast cancer

Cesar F. Ortega-Cava,[1] Srikumar M. Raja,[1,2,*] Zenab Laiq,[1] Tameka A. Bailey,[1] Haitao Luan,[1,3] Bhopal Mohapatra,[1,4] Stetson H. Williams,[1] Aaron C. Ericsson,[5] Rasna Goswami,[6] Manjari Dimri,[7] Lei Duan,[1,8] Vimla Band,[1,2,3] Mayumi Naramura,[1,2,3,*] and Hamid Band[1,2,3,4,9,10,*]

[1]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA
[2]UNMC-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA
[3]Departments of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, College of Medicine, Omaha, NE, USA
[4]Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, Omaha, NE, USA
[5]Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA
[6]Abbott Laboratories, Abbott Park, IL, USA
[7]Department of Biochemistry, George Washington University School of Medicine, Washington, DC, USA
[8]Current address for LD - Department of Radiation Oncology, Rush University Medical Center, Chicago, IL, USA
[9]Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, College of Medicine, Omaha, NE, USA
[10]Pharmacology and Experimental Neuroscience, College of Medicine, University of Nebraska Medical Center, College of Medicine, Omaha, NE, USA
Srikumar M. Raja: sraja@unmc.edu ; Mayumi Naramura: mnaramura@unmc.edu ; Hamid Band: hband@unmc.edu
*Corresponding author

Received September 12, 2011; Accepted October 20, 2011.

Abstract

Background:

Well over a quarter of human breast cancers are ErbB2-driven and constitute a distinct subtype with substantially poorer prognosis. Yet, there are substantial gaps in our understanding of how ErbB2 tyrosine kinase activity unleashes a coordinated program of cellular and extracellular alterations that culminate in aggressive breast cancers. Cellular models that exhibit ErbB2 kinase dependency and can induce metastatic breast cancer in immune competent hosts are likely to help bridge this gap.

Materials and Methods:

Here, we derived and characterized a cell line model obtained from a transgenic ErbB2/Neu-driven mouse mammary adenocarcinoma.

Results:

The MPPS1 cell line produces metastatic breast cancers when implanted in the mammary fat pads of immune-compromised as well as syngeneic immune-competent hosts. MPPS1 cells maintain high ErbB2 overexpression when propagated in DFCI-1 or related media, and their growth is ErbB2-dependent, as demonstrated by concentration-dependent inhibition of proliferation with the ErbB kinase inhibitor Lapatinib. When grown in 3-dimensional (3-D) culture on Matrigel, MPPS1 cells predominantly form

large irregular cystic and solid structures. Remarkably, low concentrations of Lapatinib led to a switch to regular acinar growth on Matrigel. Immunofluorescence staining of control vs. Lapatinib-treated acini for markers of epithelial polarity revealed that inhibition of ErbB2 signaling led to rapid resumption of normal mammary epithelium-like cell polarity.

Conclusions:

The strict dependence of the MPPS1 cell system on ErbB2 signals for proliferation and alterations in cell polarity should allow its use to dissect ErbB2 kinase-dependent signaling pathways that promote loss of cell polarity, a key component of the epithelial mesenchymal transition and aggressiveness of ErbB2-driven breast cancers.

Keywords: 3-D Matrigel, EMT, ErbB2/Her2/Neu, lapatinib, mouse models, syngeneic xenografts

BACKGROUND

ErbB2 (Her2/Neu), a member of the ErbB family of receptor tyrosine kinases (RTKs), is overexpressed and causally linked to oncogenesis in over a quarter of all cases of breast cancer; notably, ErbB2 overexpression defines a distinct molecular subtype of advanced breast cancers with an especially poor clinical prognosis.[1–3] The overexpression of ErbB2 in breast cancers has also provided an opportunity for targeted therapies that take advantage of the cell surface expression (humanized monoclonal antibodies, such as Trastuzumab) and kinase activity (kinase inhibitors, such as lapatinib) of ErbB2; both of these strategies are now in clinical use with a significant degree of success in patient management.[4–8] It is however clear that these elegant therapeutic strategies have been less successful than initially anticipated. Both primary and acquired resistance has emerged as a substantial barrier to success of targeted therapeutics.[9–12] These issues highlight the need for better understanding of how ErbB2 initiates and maintains the various hallmarks of oncogenesis, how targeted therapeutic agents produce their beneficial effects and what mechanisms contribute to their failure. Answers to these questions require appropriate experimental models that can be used for molecular, cellular and *in vivo* studies in a relatively seamless manner. Currently, few models with these attributes exist.

Much of our current understanding of signaling pathways downstream of ErbB2, mechanisms by which targeted therapeutic agents work and mechanism of resistance to these agents have been gleaned from studies of human ErbB2-overexpressing breast cancer cell lines analyzed *in vitro* and as xenograft tumors. A major weakness of these models is that *in vivo* analyses have to be carried out in immune-compromised murine hosts. It is now amply clear that components of the immune system play key negative as well as positive roles in oncogenesis.[13–16] Furthermore, immune mechanisms contribute prominently to the effectiveness of targeted therapy with humanized antibodies against ErbB2.[17–19] Thus, while human ErbB2-overexpressing tumor cell lines have provided critical *in vitro* systems to advance our understanding of ErbB2-mediated oncogenesis, these models lack key attributes relevant to oncogenesis and targeted therapy *in vivo*.

Transgenic mice that specifically overexpress ErbB2 in the mammary epithelium have provided an important complement to the xenograft-based models and have aided in analyses of the biology of ErbB2-driven oncogenesis as well as studies of targeted therapeutics.[20,21] The relatively long time to onset (many months), and variability in the onset and progression among individual animals in a cohort make these models challenging especially for studies investigating therapeutic strategies and mechanisms.

Thus, while human breast cancer cell lines together with transgenic mouse models have led to substantial improvement in our understanding of ErbB2-mediated oncogenesis, there is a substantial need for well-characterized syngeneic murine cell line models that eliminate the need for immune-

compromised hosts and exhibit strict ErbB2 dependence. Models that incorporate ease of use of cell lines together with tumorigenesis in immune-competent mouse models are therefore highly desirable to help bridge gaps in our knowledge of ErbB2-driven oncogenesis and to improve ErbB2-targeted therapies. Cell line models derived from ErbB2 transgenic mouse mammary tumors are particularly well-suited to address these needs. Prior studies have described the establishment of cell lines from ErbB2 transgenic mice that could form tumors when inoculated in mammary fat pads of immune-compromised or immune-competent mice[22–24] and in certain cases could produce lung metastases either when injected intravenously or spontaneously from the primary site,[25] thus validating the approach. However, while these cell lines have proven useful in tumor vaccine and biological studies, it has not been firmly established with these cell line models whether these are strictly dependent on ErbB2 kinase, a very desirable trait. On the other hand, it is well established that ErbB2 overexpression in the mammary epithelium promotes invasive tumors that can metastasize.[3,26,27]

Here, we describe a cell line model derived from an MMTV-ErbB2/Neu transgenic mouse mammary tumor that is capable of forming primary as well as metastatic tumors following orthotopic mammary fat pad inoculation. Importantly, 2- and 3-dimensional culture studies establish that oncogenic attributes of the cell line established are strictly dependent on the ErbB2 kinase activity. In addition, we show that inhibition of the ErbB2 kinase activity promotes a normal epithelial-like polarity in 3-dimensional culture, suggesting that continued signaling downstream of the ErbB2 kinase maintains the oncogenic trait of loss of polarity in the model described here. Thus, the present model system should allow a molecular dissection of pathways that link ErbB2 to altered epithelial polarity and epithelial mesenchymal transition as part of the metastasis program of ErbB2-driven tumors.

MATERIALS AND METHODS

Establishment of mouse mammary tumor cell lines from MMTV-ErbB2 transgenic mice

All animal-related procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines. MMTV-Neu Tg [FVB/N-Tg(MMTV-Neu)202Mul/J] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice were followed for appearance of palpable mammary tumors. Once established, tumors were aseptically resected, minced, and digested in F12 medium with 5 % FCS supplemented with 3 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) and 1.5 mg/ml trypsin (Invitrogen, Carlsbad, CA) for 2 hours at 37° C. Dissociated cells were centrifuged at 200 *g*, washed twice, resuspended in DFCI-1 medium and cultured in a humidified incubator with 5% CO2 at 37° C. Cells were released with trypsin/EDTA when confluent. After serial passaging, we obtained three independent stable cell lines named MPPS1, MPPS2 and MPPS3 (please see footnote[*]) from individual mammary tumors arising in a female MMTV-Neu transgenic female mouse. MPPS1 tumor cell line was used in the present study. The MPPS1LA sub-line was developed by adapting MPPS1 cells to grow on ultra low attachment plates (Corning, Lowell, MA) in D2 medium; D2 is a derivative of DFCI-1 medium that lacks fetal bovine serum and bovine pituitary extract.[28]

Antibodies and other reagents

The following primary antibodies were obtained, from the indicated commercial sources: rabbit anti-cNeu (ErbB2) (C-18) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antibody broadly reactive with cytokeratins (pan-keratin) from DAKO (Carpinteria, CA); mouse monoclonal anti-vimentin (clone RV202) from BD Pharmingen (San Jose, CA); mouse monoclonal anti-zona occludens 1 (ZO-1) (clone ZO-1-1A12) from Invitrogen Inc (Carlsbad, CA); mouse monoclonal anti-heat shock cognate protein of 70 kDa (Hsc70) (B-6) from Santa Cruz Biotechnology; and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (clone 6C5) from Chemicon Int. (Temecula, CA). The mouse monoclonal anti-phosphotyrosine (anti-pY; 4G10)[29] was kindly provided by Dr. Brian Druker (Oregon

Health and Science University, Portland, OR). The ErbB receptor tyrosine kinase inhibitor lapatinib [30,31] was from LC laboratories (Woburn, MA) and was stored as a 1mM solution in dimethyl sulfoxide (DMSO).

Assessment of cell proliferation

To quantify the effects of lapatinib on cell proliferation, cells were seeded in triplicate at 0.5×10^4/well in 96-well plates and incubated in the presence of the indicated concentrations of lapatinib. Seventy-two hours later, the culture medium was removed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.5 mg/ml for 4 hours. The cells were lysed by adding SDS to 1 % and absorbance was read at 570 nm. The % of cells alive at the end of the drug treatment, based on the absorbance of the formazan was calculated as $[(O.D_{570})^{treated}/(O.D_{570})^{untreated}] \times 100$.[32]

Three-dimensional (3-D) Matrigel culture

Laminin-rich basement membrane matrix (Matrigel®) for 3-D cultures was obtained from BD Biosciences (San Jose, CA). Trypsin/EDTA-released and washed cells were suspended in 2% (v/v) Matrigel in DFCI-1 medium and seeded over a layer of polymerized 100% Matrigel at 3×10^4 per cm^2 in 6- or 24-well plates or in 8-well chamber slides (for Confocal microscopy), essentially as described previously.[33] For growth on Ultra Low Attachment culture ware (Corning), cells were suspended in 4% (v/v) Matrigel/DFCI-1 medium mix.

Immunofluorescence microscopy

Immunofluorescence staining of cells grown in 3-D cultures was performed essentially as described.[33] In brief, the cells were fixed in 4% paraformaldehyde in PBS, washed with PBS/100 mM glycine and permeabilized in 0.5% Triton X-100 for 5 minutes. After washing, cells were blocked with 10% goat serum and then incubated with anti-ErbB2 (1/500), pan-anti-cytokeratin (1/5000), anti-vimentin (1/200) or anti-ZO-1 (1/200) for 1 hour at room temperature. After 3 washes, species-appropriate secondary antibodies conjugated with Alexa 488 or Alexa 546 (Invitrogen) was used to visualize the bound primary antibody. Alexa Fluor 594 phalloidin (Invitrogen) was mixed with the secondary antibody for visualization of polymerized actin. Vectashield Hard Set mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used for mounting. Images were captured under a 63x oil immersion lens using LSM510 or LSM410 fluorescence confocal microscopes (Carl Zeiss) and processed with Zeiss LSM Image Browser version 4.2.0.121. Experiments were repeated at least thrice and an isotype-matched IgG was used as a negative control.

In vivo tumorigenicity assay

Athymic nude mice (Nu/Nu) were from Charles River Laboratories (Wilmington, MA). FVB/NJ mice were from Jackson Laboratory (Bar Harbor, ME). Seven-week old female mice were anesthetized and kept sedated using Ketamine along with Xylazine to directly visualize the fourth mammary gland through a small skin incision. 10^6 cells in 20 μl of DFCI medium were injected into the mammary fat pad using a Hamilton syringe. The tumor size was measured weekly using vernier calipers. Tumor volumes were calculated as: ½ larger diameter × (smaller diameter)^2.[34] Animals were euthanized and necropsies were performed when tumors reached a size of approximately 1,000 mm^3. Primary tumors and lungs were harvested for histopathology. At least five animals were used per experimental group.

RESULTS

Derivation and characterization of an ErbB2-driven mouse mammary tumor cell line MPPS1

Three separate primary mammary tumors (PT1, 2 and 3) were resected from an eleven-month old virgin female MMTV-Neu Tg mouse that developed multiple mammary tumors. Independent cell lines established from these three tumors were designated MPPS1, MPPS2 and MPPS3; MPPS1 line was characterized in detail. These cells exhibit robust and continuous growth as monolayers in DFCI-1 medium, and maintain high levels of ErbB2/Neu comparable to those in the primary tumors, as demonstrated using Western blotting [Figure 1a]. We did not notice any significant loss of ErbB2/Neu expression as has been reported by Guo *et al.* 2006 with primary tumor cells derived from MMTV-Neu (YD) mice upon prolonged *in vitro* propagation;[35] in fact, the levels of ErbB2 in MPPS1 cells remained unchanged when these were maintained continuously over 20 passages under our culture conditions [Figure 1a]. As expected for an epithelial cell-derived tumor cell line, MPPS1 cells stained positive with a pan-cytokeratin antibody while they did not stain significantly with an anti-vimentin antibody [Figure 1b]. In addition, smooth muscle actin was not detectable by immunostaining (data not shown). The cells stained prominently with an anti-ErbB2 antibody with the ErbB2 staining predominantly localized at the plasma membrane, giving a honey-comb appearance in confluent cultures [Figure 1c]. Addition of the EGFR/ErbB2 dual kinase inhibitor lapatinib in the culture medium led to a dose-dependent inhibition of cell proliferation, with an IC_{50} of 0.125 ± 0.001 μM [Figure 1d], indicating that MPPS1 cells are fully dependent on ErbB2 activity for cell proliferation. The ability of lapatinib to inhibit the ErbB2 kinase activity was shown by a concentration-dependent reduction in auto-phosphorylated ErbB2 levels [Figure 1e]. As reported by Scaltriti *et al*[36] in human ErbB2-overexpressing breast cancer cell lines, we also observed that lapatinib treatment led to accumulation of ErbB2 proteins [Figure 1 e; note the dose-dependent increase in the intensity of ErbB2 signal following lapatinib treatment]; this is likely due to reduced degradation of ErbB2 upon inhibition of its kinase activity.

Orthotopic implantation of MPPS1 cells in the mouse mammary gland results in tumors at the implant site as well as distant metastases

To assess whether ErbB2-dependent MPPS1 cells retain their oncogenic potential, we assessed their ability to form tumors at the site of orthotopic implantation in the mouse mammary gland as well as their ability to produce distant metastases. While implantation in syngeneic murine hosts is expected to allow tumor growth, studies of implanted MMTV-ErbB2/Neu transgenic tumors have shown that the "take rate" is better in ErbB2 transgene-carrying host mice (implanted prior to tumor development) than in naïve syngeneic hosts; this trait is likely related to induced immune tolerance mechanisms that are not fully elucidated[37,38] Therefore, we tested the tumor-forming capabilities of MPPS1 cells in female mice of three host backgrounds: immune-compromised nude mice; young isogenic MMTV-Neu Tg mice before any evidence of endogenous tumors; the FVB/NJ strain mice (same background as the tumor-bearing mice from which MPPS1 line was derived). Table 1 lists the various conditions used for orthotopic implantation into the mammary fat pad. Consistent with the reported facilitation of tumor formation in MMTV-Neu transgenic mice, 100 % of the nude and MMTV-Neu Tg recipients developed tumors around the site of injection [Table 1]; the tumors reached a volume of approximately 1,000 mm^3 in 9 to 10 weeks in both recipient strains [Figure 2a]. Tumors also formed when MPPS1 cells were orthotopically implanted in the mammary fat pad of FVB/NJ mice; however, 10 times more cells were required for orthotopic tumor growth compared to the other two recipient strains as shown in Table 1. Upon necropsy, multiple tumor nodules were observed in various parts of the lungs of tumor-bearing animals in all three recipient strains [Figure 2b].

Histopathological comparison of primary tumors and lung metastasis between one of the two mice that developed tumors in the FVB/NJ background with that from Nu/Nu [Figure 3a] showed similar features with ductal/glandular structures consistent with a well-differentiated adenocarcinoma. No significant differences were observed among MMTV-Neu Tg and Nu/Nu in the extent of metastatic lesions within

the lungs [Figures 2b and 3b]. Together, these analyses demonstrate that transgenic ErbB2-overexpressing murine mammary tumor-derived cell line MPPS1 retains its oncogenic ability with a unique feature that orthotopic implantation in the mammary gland is associated with rapid metastasis to distant sites.

MPPS1 cell line as a model of persistent ErbB2 kinase activity-dependent alterations in epithelial cell polarity and lumen-filling hyper-proliferation phenotypes

Studies of ErbB2-driven human breast cancers as well as transgenic mouse models have shown that loss of epithelial cell polarity is a key event in oncogenic progression and a harbinger of epithelial-mesenchymal transition (EMT)[39–42] that has been linked to more aggressive tumor formation and more recently to cancer stem cell phenotype.[43] Given the ability of MPPS1 cells to metastasize from orthotopic primary tumors in the mammary gland, we wished to further characterize the alterations in the epithelial cell-cell junctions and polarity of these cells as well as the relationship of these traits with activity of the driver oncogene ErbB2. We and others have previously demonstrated that overexpression of ErbB family receptors in non-tumorigenic human mammary epithelial cells leads to alterations in cellular polarity which allows hyper-proliferation when cells are cultured under polarity-inducing 3-D Matrigel culture system.[33,39] Therefore, we carried out the characterization of cellular structures formed by MPPS1 cells when grown in 3-D culture. Many of these analyses were done with a low attachment-adapted and morphologically more homogenous subline (MPPS1LA) which was more suitable for immunofluorescence studies; The MPPS1LA cells expressed ErbB2 at levels comparable to that of ErbB2 in the parental MPPS1 cell line (data not shown) and were ErbB2-dependent as seen by the effect of lapatinib treatment on their growth [Figures 1d and 5c].

When seeded in Matrigel, MPPS1LA cells formed irregular spherical structures without any evident lumen [Figure 4a, left panel]; however, branched structures which are seen with *in vitro* ErbB2-transformed immortal human mammary epithelial cells[33,39] were rare. Immunofluorescence staining using established markers demonstrated a complete lack of epithelial cell polarity in 3D-cultured MPPS1LA cells. For example, the tight junction marker ZO-1 that is normally concentrated at the sub-apical cell-cell junctions (of a single layered epithelium of acini with a lumen)[33,44] is instead seen diffusely localized throughout the disorganized cellular masses that lack any semblance of a lumen [Figure 4b]. Staining for F-actin (phalloidin stained), normally localized to the apical cortical region of polarized mammary epithelial cells to form a continuous circular collar around the acini,[33,44] is also diffusely present throughout the disorganized cellular structures [Figure 4b and c]. Staining for E-cadherin [Figure 4c], which in polarized mammary epithelial cells is basolaterally localized to adherens junctions with little intracellular staining,[33,44] showed weak and relatively diffuse staining throughout the cells with many intracellular punctae and no discernible adherens junction-like staining. These analyses revealed that MPPS1 cells fail to polarize when grown in 3-D Matrigel culture, a feature consistent with their transformed phenotype.[39,45]

Given the dependence of MPPS1 cell proliferation on ErbB2 when analyzed in 2-D culture [Figure 1d], we utilized lapatinib treatment to assess if the abnormal polarity and the associated hyper-proliferation of MPPS1 cells in 3-D culture are also causally linked to continued kinase activity-dependent signaling of the driver oncogene ErbB2. First, we looked at the dose-response and kinetics of lapatinib-induced reduction in pY-ErbB2 levels in cells grown on 3-D Matrigel cultures. Immunoblotting of cell lysates obtained from 3-D cultures demonstrated that lapatinib treatment indeed induced a dose-dependent reduction in phosphorylated (active) ErbB2 [Figure 5a] with a concomitant increase in the total level of ErbB2 when examined after 48 h of treatment, as seen in cells treated with lapatinib in 2-D culture [Figure 1e]. Notably, the effects of lapatinib were relatively quick, as seen by an essentially complete loss of pY-ErbB2 signals within 6h of treating 3-D cultures with 100 nM lapatinib [Figure 5b]. Importantly,

lapatinib treatment was associated with expected reduction in phosphorylated (active) pools of AKT and ERK1/2 without a change in total AKT or ERK1/2 levels [Figure 5b], indicating that lapatinib treatment at relatively low concentrations indeed abrogated ErbB2 kinase-dependent downstream signaling.

Addition of lapatinib to 3-D cultures led to a concentration-dependent reduction in the number of irregular acini and their overall size. At high concentrations (0.5 to 1 μM), the cell structures tended to disintegrate consistent with cytotoxic effects [Figure 5c]. Notably however, cultures treated with intermediate concentrations (0.1 to 0.25 μM) of lapatinib showed a predominance of regular acinus-like structures with lumens [Figure 5c and 6a], suggesting a reversal of abnormal polarity. Staining with markers of polarity demonstrated that this was indeed the case: as is evident in Figure 6b, a lapatinib-treated 3-D cultures showed a predominance of single epithelial layer-lined acinar structures with a clear lumen (as seen with DAPI staining near the periphery and lack of nuclei in the middle). Importantly, these acini showed clear and well-formed tight junctions (ZO-1 staining), sub-apical actin bands (phalloidin staining), apical GM130 and E-cadherin localization in basolateral adherens junctions [Figures 6b–d]. Biochemically, lapatinib treatment led to a concentration-dependent accumulation of E-cadherin protein levels [Figure 6e]. These results suggest that continuous signaling downstream of an active ErbB2 kinase produces the abnormal polarity and associated lumen-filling hyper-proliferation phenotypes of MPPS1 cells.

The ability of MPPS1 cells to attain normal epithelial-like polarity and growth characteristics upon ErbB2 kinase inhibition, as early as 12-24 hours after initiating treatment [Figure 5b], prompted us to ask if the lapatinib effect was terminal or could be reversed upon washout of the drug. Treatment of 3-D cultures with 0.1 lapatinib for 24 h was associated with a reduction in pY-ErbB2 levels as expected, as well as the appearance of regular acinar structures [Figures 7a and b]. Washout of lapatinib led to a slow but almost complete recovery of pY-ErbB2 signals [Figure 7a] and the cultures concomitantly resumed proliferation regaining a predominance of abnormal cellular structures that lacked lumens [Figure 7b]. These results indicate that MPPS1 cells offer a reversible system to link ErbB2 signals with specific oncogenic traits.

DISCUSSION

Cellular models have provided important clues into mechanisms by which the human cancer oncogene ErbB2, which is causally linked to over a quarter of invasive breast cancers, unleashes the program of oncogenic transformation of mammary epithelial cells. Yet, there is a lack of facile cellular models that allow biochemical and molecular analyses over a wide range of oncogenic transitions from relatively normal cellular behavior to metastasis. Furthermore, full dependence of oncogenic progression on ErbB2 kinase-dependent signaling would help enhance the appeal of such cellular models. Recent studies have focused on either human cancer-derived ErbB2-overexpressing cell lines or on deliberate transformation of non-tumorigenic immortal human mammary epithelial cell lines; however, the former often exhibit variable dependence on ErbB2 signals while the latter typically show only subtle oncogenic transformation discernible only using sensitive systems such as 3-D culture in Matrigel; such transformed cells often fail to make tumors in xenotransplanted mice and typically do not exhibit metastatic behavior.[33,46,47] As ErbB2 transgenes reproducibly lead to mammary tumors that metastasize,[25,48] cellular models from such mice could bridge the current gap in our understanding of mechanisms of ErbB2-driven oncogenesis. While several previous attempts have been made to establish cell line models from ErbB2 transgenic mice, these cell systems have not been well characterized as suitable models of critical oncogenic traits associated with ErbB2-mediated oncogenic transformation. Here, we have generated and characterized a murine ErbB2 transgene (rat ErbB2/Neu)-induced mammary tumor cell line system whose attributes make it a desirable cellular model to help bridge gaps in our knowledge of mechanisms by which ErbB2 signals unleash the oncogenic program of mammary

epithelial cells.

The MMTV-Neu Tg mouse-derived mammary tumor cell line MPPS1 not only produces primary tumors when orthotopically implanted in the mammary gland but it also exhibits rapid metastatic ability as shown by the presence of lung metastasis in essentially all primary tumor-bearing animals. While rapid tumor growth and metastatic behavior by itself is not unique, it is rather notable that both the primary and metastatic tumors maintained relatively well-differentiated tumor histology [Figure 3a]. More importantly, *in vitro* characterization demonstrated that MPPS1 cells proliferate continuously but unlike previous reports[35] the levels of ErbB2 are not lost in this cell line during culture [Figure 1a]. This in itself suggested the potential dependence of oncogenic traits of MPPS1 cells on continued ErbB2 expression and its signaling. Indeed inhibition of ErbB2 kinase activity with lapatinib demonstrated that cell proliferations in 2-D and 3-D culture is fully dependent on continued activity of ErbB2. Thus, MPPS cells should provide a useful model to investigate pathways that provide an essential link between ErbB2 signals and deregulation of cell cycle and associated programs such as cancer cell metabolism, especially under 3-D culture conditions that mimic tissue architecture.

As previous studies have demonstrated, the pathways of normal cellular proliferation and differentiation as well as their aberrations during oncogenesis are dramatically influenced by the interaction of cells with each other and with the microenvironment as well as the nature of matrix.[49,50] Thus, a tumor cell line model that exhibits complete abrogation of cell polarity and lumen formation, important attributes of normal mammary epithelial cells in tissues as well as in 3-D culture, when the driver oncogene (ErbB2) is active but rapidly reverses these attributes towards a normal cell behavior [Figures 6a–e] when oncogenic signals are turned off should provide a valuable system to mechanistically trace upstream ErbB2 signals to regulation of mammary epithelial morphogenesis and its alterations in breast cancer. Our initial studies indicating that MPPS1 cell system allows reversion of oncogenic traits upon lapatinib inhibition as well as resumption of oncogenic traits upon lapatinib washout make the current system particularly attractive.

Complementing the *in vitro* attributes, it is notable that metastatic tumors developed with a short latency after orthotopic implantation in host mice, and this could be achieved in immune competent hosts as well albeit more tumor cells were needed to develop tumors in naïve parental strain. As antibody-dependent targeted therapy has become an essential component of therapy for ErbB2-driven breast cancers, models in which metastatic disease develops reproducibly and quickly are needed to investigate mechanisms of therapeutic response as well as resistance using *in vivo* settings with an intact immune system, something lacking with xenograft models. The current system should be very useful in this regard.

It is of note that tumor growth and metastasis in syngeneic (MMTV-Neu Tg) females were rapid, highly reproducible and comparable to tumors seen in immune-compromised nude mice. However these attributes were somewhat slower and less robust in parental FVB/NJ mice. This observation is consistent with previous reports that rat ErbB2/Neu oncogene is immunogenic in non-transgenic FVB mice.[37,38] However, the ability of MPPS1 cells to reproducibly grow and metastasize spontaneously from the primary tumor in immune competent MMTV-Neu Tg mice should still provide a practical immune-competent model to study tumor progression and therapeutic mechanisms. As the latency of spontaneous tumors in the MMTV-Neu Tg mice was as reported previously (about 6 months),[27,51] yet the implanted MPPS1 tumor grew and metastasized before these mice had attained this age (about 7 weeks of age at implant plus 10-11 weeks of tumorigenesis for a total of 17-18 weeks of age at the completion of the experiment), it is unlikely that the tumors at the implant site or metastases in the lung represent spontaneous tumors. This interpretation is supported by lack of any tumors in non-implanted mammary glands of recipient mice. We have recently developed GFP-expressing MPPS1 cells which

should allow future studies to unequivocally distinguish implanted tumors and their metastases from endogenous tumors.

Recent years have seen an expansion of high throughput technologies that have begun to yield important mechanistic clues relevant to biology of cancer as well as mechanisms of therapeutic response and resistance. In initial studies, we have observed that MPPS1 cell system is easily adaptable for gene transfer experiments and cell-based assays. Together with the ErbB2-dependent 3-D culture traits and the ability to rapidly induce primary and metastatic tumors in syngeneic hosts, the MPPS1 cell line should be easily adaptable for linked *in vitro* and *in vivo* high throughput studies within a single cell model.

CONCLUSIONS

Collectively, the MPPS1 cell model of ErbB2-driven breast cancer described here demonstrates that loss of cellular polarity and lumen filling in 3-D environment requires continuous ErbB2 signals. Our results suggest that the relatively unique cellular model described here should help in molecular dissection of oncogenesis as well as mechanisms of therapeutic response and resistance in ErbB2-driven primary and metastatic breast cancer using *in vivo* studies in immune-competent animals that can be completed in a short time and in a cost effective manner. This cellular model should also provide a tool to dissect signaling pathways linking upstream ErbB2 signaling with early as well as late oncogenic traits using high throughput approaches that exploit the ability to carry out *in vitro* and *in vivo* tumor progression studies in a single cellular system.

List of Abbreviations used

DAPI - 4',6-diamidino-2-phenylindole; DFCI-1 – mammary epithelial cell culture media named after Dana Farber Cancer Institute; D2 - a derivative of DFCI-1 medium that lacks fetal bovine serum and bovine pituitary extract; DMSO – Dimethyl Sulfoxide; EMT – Epithelial to Mesenchymal Transition; ErbB2 (Her2/Neu) – Epidermal Growth Factor Receptor 2; EDTA – Ethylene Diamine Tetra Acetic acid; FCS – Fetal Calf Serum; GAPDH – Glyceraldehyde phosphate dehydrogenase; Hsc70 – Heat shock cognate protein 70; MPPS1 (and MPPS2 and 3) – ErbB2/Neu driven mammary epithelial cell line derived from primary tumors from MMTV-Neu Tg mice; MPPS1LA – MPPS1 derived cell line enriched for growth in low attachment cell culture plates; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PT1, 2 and 3 - Primary Tumor 1, 2 and 3; SDS – Sodium dodecyl sulfate; Tg – Transgenic; ZO-1 – Zona Occludens 1

AUTHOR CONTRIBUTIONS

The study was conceived and directed by HB, VB, MN, SMR and CFO-C. The experimental work was executed by CFO-C with contributions from ZL, RG, SHW, MD, LD, BM, HL, TAB, MN and SMR. ACE helped with the histopathology-related studies. CFO-C wrote the first draft that was edited, into final manuscript by HB, SMR and MN. TAB, HL and BM also helped organizing the manuscript figures and literature for citations.

AUTHOR'S PFOFILE

Dr. Mayumi Naramura, Eppley Institute for Research in Cancer and Allied Diseases, Departments of Genetics, Cell Biology & Anatomy, UNMC-Eppley Cancer Center, College of Medicine, University of Nebraska Medical Center, Omaha, NE

Dr. Srikumar M Raja, Eppley Institute for Research in Cancer and Allied Diseases, UNMC-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE

Prof. Hamid Band, Eppley Institute for Research in Cancer and Allied Diseases; Departments of Genetics, Cell Biology and Anatomy, Biochemistry and Molecular Biology, Pathology and Microbiology, and Pharmacology and Experimental Neuroscience, College of Medicine; and UNMC-Eppley Cancer Center, College of Medicine, University of Nebraska Medical Center, Nebraska Medical Center Omaha, NE

ACKNOWLEDGMENTS

This work was supported by the NIH grants CA87986, CA99163, CA105489 and CA116552, to HB, and CA96844 and CA81076 to VB; Department of Defense Breast Cancer Research Grants W81XWH-11-1-0166 (HB), W81XWH-07-1-0351 and W81XWH-11-1-0171 (V.B) and W81XWH-10-1-0740 (M.N.); the National Cancer Institute (NCI 5U01CA151806-02) (HB and SMR); Nebraska Department of Health and Human Services (LB-506 grant to SMR); and Nebraska Center for Nanomedicine-Center for Biomedical Research Excellence (NCN-COBRE; seed grant to SMR). TAB was a trainee under an NCI Cancer Biology Training Grant (T32 CA 009476) and is currently a Susan G. Komen Foundation post-doctoral fellow (KG091363). The investigators acknowledge support from the Department of Medicine, Evanston Northwestern Healthcare Research Institute (now NorthShore University HealthSystem Research Institute), Northwestern University, Evanston, IL, where these studies were initiated. We thank Dr. Dr. Brian Druker (Oregon Health and Science University) for mAb 4G10; and Janice Taylor and James Talaska of the confocal facility for technical assistance. The Confocal Microscopy Core at UNMC is supported by the Nebraska Research Initiative and the NCI CCSG to UNMC-Eppley Cancer Center. We thank members of the Band laboratories for helpful suggestions and discussion.

Footnotes

*The author CFO-C named the cell lines as MPPS as a tribute to the life of his late wife Merlyn Patricia Pozada Sanchez.

REFERENCES

1. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52. [PubMed: 10963602]

2. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98:10869–74. [PMCID: PMC58566] [PubMed: 11553815]

3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177–82. [PubMed: 3798106]

4. Baselga J. Treatment of HER2-overexpressing breast cancer. Ann Oncol. 2010;21(Suppl 7):vii36–40. [PubMed: 20943641]

5. Di Cosimo S, Baselga J. Management of breast cancer with targeted agents: importance of heterogeneity. [corrected] Nat Rev Clin Oncol. 2010;7:139–47. [PubMed: 20125090]

6. Di Cosimo S, Baselga J. Targeted therapies in breast cancer: where are we now? Eur J Cancer. 2008;44:2781–90. [PubMed: 19013786]

7. Nahta R, Esteva FJ. Trastuzumab: triumphs and tribulations. Oncogene. 2007;26:3637–43. [PubMed: 17530017]

8. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol. 2006;3:269–80.

[PubMed: 16683005]

9. Nahta R, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. Breast Cancer Res. 2006;8:215. [PMCID: PMC1797036] [PubMed: 17096862]

10. Gajria D, Chandarlapaty S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. Expert Rev Anticancer Ther. 2011;11:263–75. [PMCID: PMC3092522] [PubMed: 21342044]

11. Garrett JT, Arteaga CL. Resistance to HER2-directed antibodies and tyrosine kinase inhibitors: Mechanisms and clinical implications. Cancer Biol Ther. 2011;11:793–800. [PMCID: PMC3230295] [PubMed: 21307659]

12. Spector NL, Blackwell KL. Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol. 2009;27:5838–47. [PubMed: 19884552]

13. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7. [PMCID: PMC2803035] [PubMed: 12490959]

14. Tlsty TD, Coussens LM. Tumor stroma and regulation of cancer development. Annu Rev Pathol. 2006;1:119–50. [PubMed: 18039110]

15. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006;6:24–37. [PubMed: 16397525]

16. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res. 2007;9:212. [PMCID: PMC2206719] [PubMed: 17705880]

17. Lazar GA, Dang W, Karki S, Vafa O, Peng JS, Hyun L, et al. Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci U S A. 2006;103:4005–10. [PMCID: PMC1389705] [PubMed: 16537476]

18. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. Nat Med. 2000;6:443–6. [PubMed: 10742152]

19. Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. Exp Hematol. 1999;27:1533–41. [PubMed: 10517495]

20. Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ. Insights from transgenic mouse models of ERBB2-induced breast cancer. Nat Rev Cancer. 2007;7:389–97. [PubMed: 17446858]

21. Pegram M, Ngo D. Application and potential limitations of animal models utilized in the development of trastuzumab (Herceptin): a case study. Adv Drug Deliv Rev. 2006;58:723–34. [PubMed: 16876287]

22. Sacco MG, Gribaldo L, Barbieri O, Turchi G, Zucchi I, Collotta A, et al. Establishment and characterization of a new mammary adenocarcinoma cell line derived from MMTV neu transgenic mice. Breast Cancer Res Treat. 1998;47:171–80. [PubMed: 9497105]

23. Sacco MG, Faggioli F, Soldati S, Gribaldo L, Collotta A, Pariselli F, et al. Establishment and characterization of new mammary adenocarcinoma cell lines derived from double transgenic mice expressing GFP and neu oncogene. Cell Prolif. 2006;39:611–22. [PubMed: 17109643]

24. Campbell MJ, Wollish WS, Lobo M, Esserman LJ. Epithelial and fibroblast cell lines derived from a

spontaneous mammary carcinoma in a MMTV/neu transgenic mouse. In Vitro Cell Dev Biol Anim. 2002;38:326–33. [PubMed: 12513120]

25. Tan W, Zhang W, Strasner A, Grivennikov S, Cheng JQ, Hoffman RM, et al. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. Nature. 2011;470:548–53. [PMCID: PMC3166217] [PubMed: 21326202]

26. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989;244:707–12. [PubMed: 2470152]

27. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A. 1992;89:10578–82. [PMCID: PMC50384] [PubMed: 1359541]

28. Band V, Zajchowski D, Swisshelm K, Trask D, Kulesa V, Cohen C, et al. Tumor progression in four mammary epithelial cell lines derived from the same patient. Cancer Res. 1990;50:7351–7. [PubMed: 1977518]

29. Druker BJ, Mamon HJ, Roberts TM. Oncogenes, growth factors, and signal transduction. N Engl J Med. 1989;321:1383–91. [PubMed: 2682241]

30. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res. 2006;66:1630–9. [PubMed: 16452222]

31. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene. 2002;21:6255–63. [PubMed: 12214266]

32. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, et al. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 1988;48:4827–33. [PubMed: 3409223]

33. Dimri M, Naramura M, Duan L, Chen J, Ortega-Cava C, Chen G, et al. Modeling breast cancer-associated c-Src and EGFR overexpression in human MECs: c-Src and EGFR cooperatively promote aberrant three-dimensional acinar structure and invasive behavior. Cancer Res. 2007;67:4164–72. [PubMed: 17483327]

34. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol. 1989;24:148–54. [PubMed: 2544306]

35. Guo W, Pylayeva Y, Pepe A, Yoshioka T, Muller WJ, Inghirami G, et al. Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. Cell. 2006;126:489–502. [PubMed: 16901783]

36. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28:803–14. [PubMed: 19060928]

37. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, et al. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. Cancer Res. 2000;60:3569–76. [PubMed: 10910070]

38. Lin CC, Chou CW, Shiau AL, Tu CF, Ko TM, Chen YL, et al. Therapeutic HER2/Neu DNA vaccine inhibits mouse tumor naturally overexpressing endogenous neu. Mol Ther. 2004;10:290–301. [PubMed: 15294176]

39. Aranda V, Haire T, Nolan ME, Calarco JP, Rosenberg AZ, Fawcett JP, et al. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. Nat Cell Biol. 2006;8:1235–45. [PubMed: 17060907]

40. Jenndahl LE, Isakson P, Baeckstrom D. c-erbB2-induced epithelial-mesenchymal transition in mammary epithelial cells is suppressed by cell-cell contact and initiated prior to E-cadherin downregulation. Int J Oncol. 2005;27:439–48. [PubMed: 16010426]

41. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell. 2005;8:197–209. [PubMed: 16169465]

42. Khoury H, Dankort DL, Sadekova S, Naujokas MA, Muller WJ, Park M. Distinct tyrosine autophosphorylation sites mediate induction of epithelial mesenchymal like transition by an activated ErbB-2/Neu receptor. Oncogene. 2001;20:788–99. [PubMed: 11314013]

43. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704–15. [PMCID: PMC2728032] [PubMed: 18485877]

44. Duan L, Chen G, Virmani S, Ying G, Raja SM, Chung BM, et al. Distinct roles for Rho versus Rac/Cdc42 GTPases downstream of Vav2 in regulating mammary epithelial acinar architecture. J Biol Chem. 2010;285:1555–68. [PMCID: PMC2801280] [PubMed: 19826000]

45. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol. 2001;3:785–92. [PMCID: PMC2952547] [PubMed: 11533657]

46. Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, et al. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. 2001;15:50–65. [PMCID: PMC312602] [PubMed: 11156605]

47. Stiles CD, Desmond W, Chuman LM, Sato G, Saier MH. Growth control of heterologous tissue culture cells in the congenitally athymic nude mouse. Cancer Res. 1976;36:1353–60. [PubMed: 1260760]

48. Chen H, Lee JS, Liang X, Zhang H, Zhu T, Zhang Z, et al. Hoxb7 inhibits transgenic HER-2/neu-induced mouse mammary tumor onset but promotes progression and lung metastasis. Cancer Res. 2008;68:3637–44. [PubMed: 18463397]

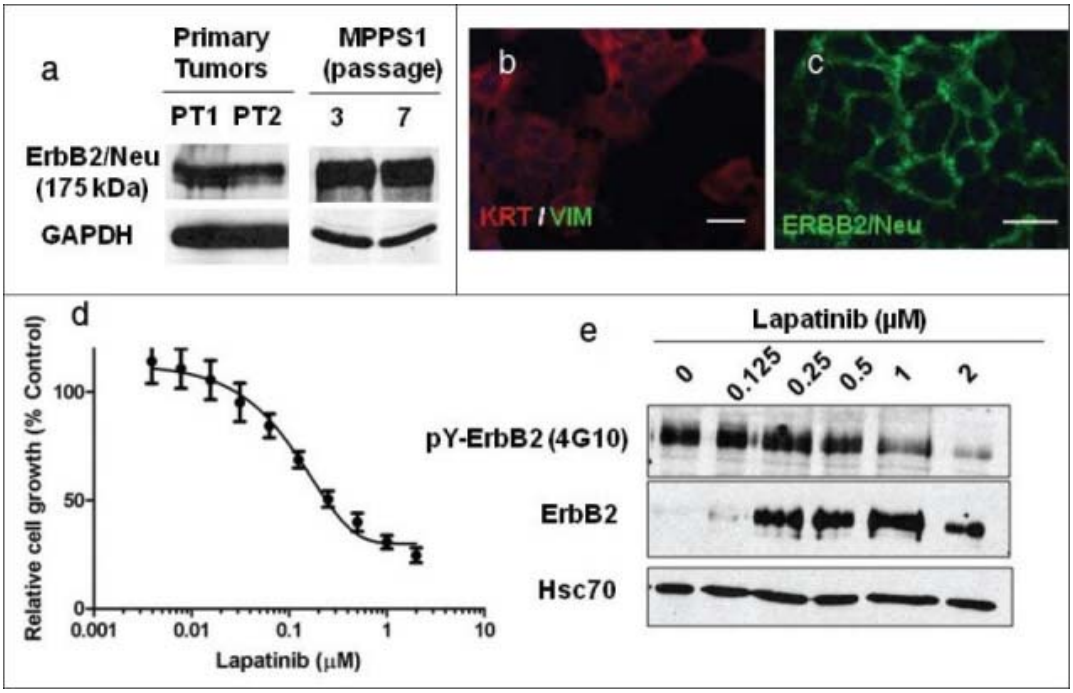
49. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat Med. 2011;17:320–9. [PubMed: 21383745]

50. Polyak K, Kalluri R. The role of the microenvironment in mammary gland development and cancer. Cold Spring Harb Perspect Biol. 2010;2:a003244. [PMCID: PMC2964182] [PubMed: 20591988]

51. Li B, Rosen JM, McMenamin-Balano J, Muller WJ, Perkins AS. Neu/ErbB2 cooperates with p53-172H during mammary tumorigenesis in transgenic mice. Mol Cell Biol. 1997;17:3155–63. [PMCID: PMC232168] [PubMed: 9154814]

Figures and Tables

Figure 1



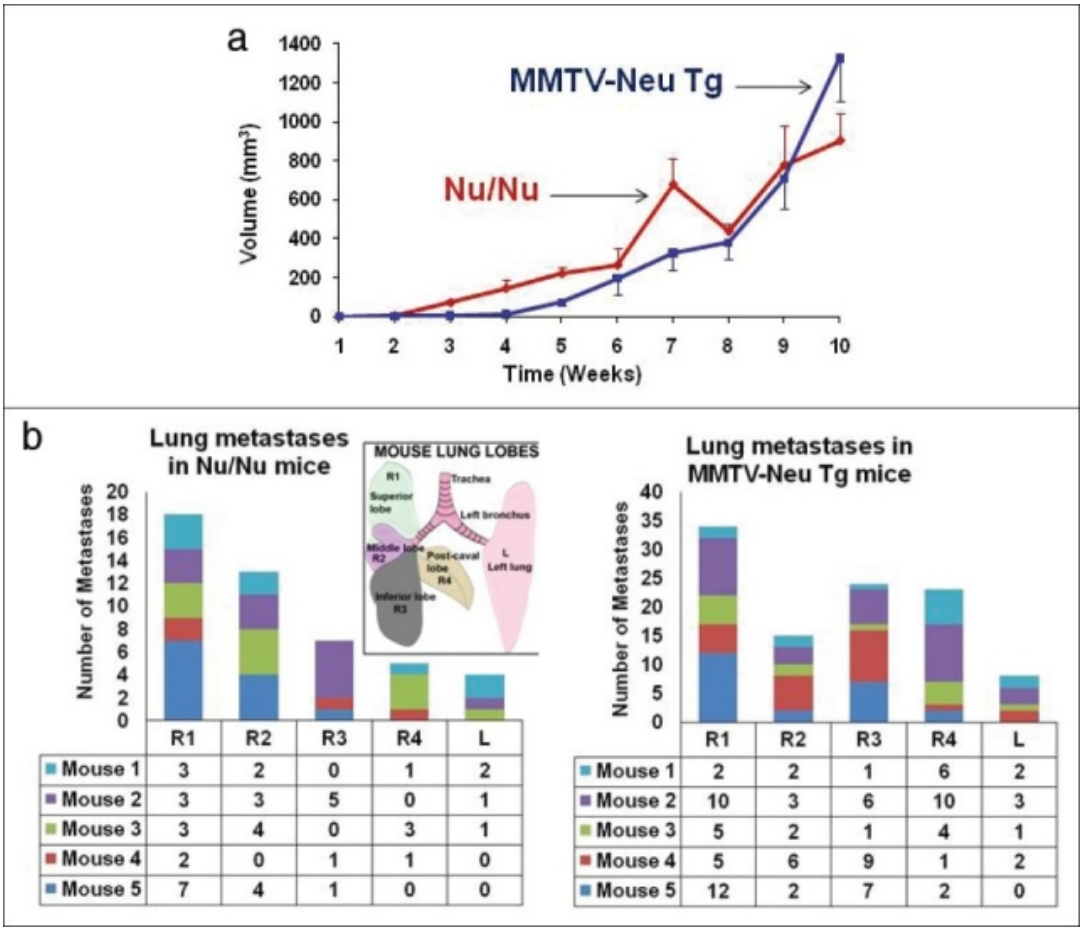
Characterization of ErbB2/Neu-driven mouse mammary tumor cell line (MPPS1): (a) Western blots comparing the expression of ErbB2/Neu in primary mammary tumors (PT1, PT2) from MMTV-Neu Tg mouse to the levels at the indicated passages of MPPS1 cell line (derived from PT1). 100 μg of total protein was loaded per lane. GAPDH is used as a loading control. (b, c) MPPS1 cells grown on glass coverslips were fixed and stained with pan-keratin (KRT), vimentin (VIM) and anti-ErbB2 (ErbB2/Neu) antibodies and detected with fluorochrome-conjugated secondary antibodies. The confocal image shows pan-keratin stained in red and vimentin in green; Scale bars in B and C are 20 μm in length. (d) Sensitivity of MPPS1 to growth inhibition by lapatinib - MPPS1 cells plated in 96-well plates as described in the methods section were treated with an increasing concentrations of lapatinib for 72 hours. The % of viable cells was estimated based on MTT assay. The calculated IC50 value (using Graphpad Prizm software) was 0.125 ± 0.001μM. (e) Concentration-dependent decrease in pY-ErbB2 levels in MPPS1 cells following treatment with lapatinib at the indicated concentrations. Cells were treated with the indicated concentrations of lapatinib for 48 hours. The reduction in pY-ErbB2 levels were assessed by SDS-PAGE/Western blotting analysis from equal amount of total protein lysates (100 μg). Hsc70 was used as loading control.

Table 1

Mouse strain	Number of implanted cells	Mice (n)	Mice with tumors	% Mice with Tumors
FVB/NJ (parental)	10×10^6	6	2	33.3
FVB/NJ (parental)	1×10^6	5	0	0
FVB/N-Tg (MMTVneu)	1×10^6	5	5	100
Nu/Nu	1×10^6	5	5	100
Nu/Nu	0.5×10^6	5	4	80
Nu/Nu	50,000	5	2	40

Host mouse strain-dependent tumor development following implantation of MPPS1 cell line in the mammary fat pad

Figure 2

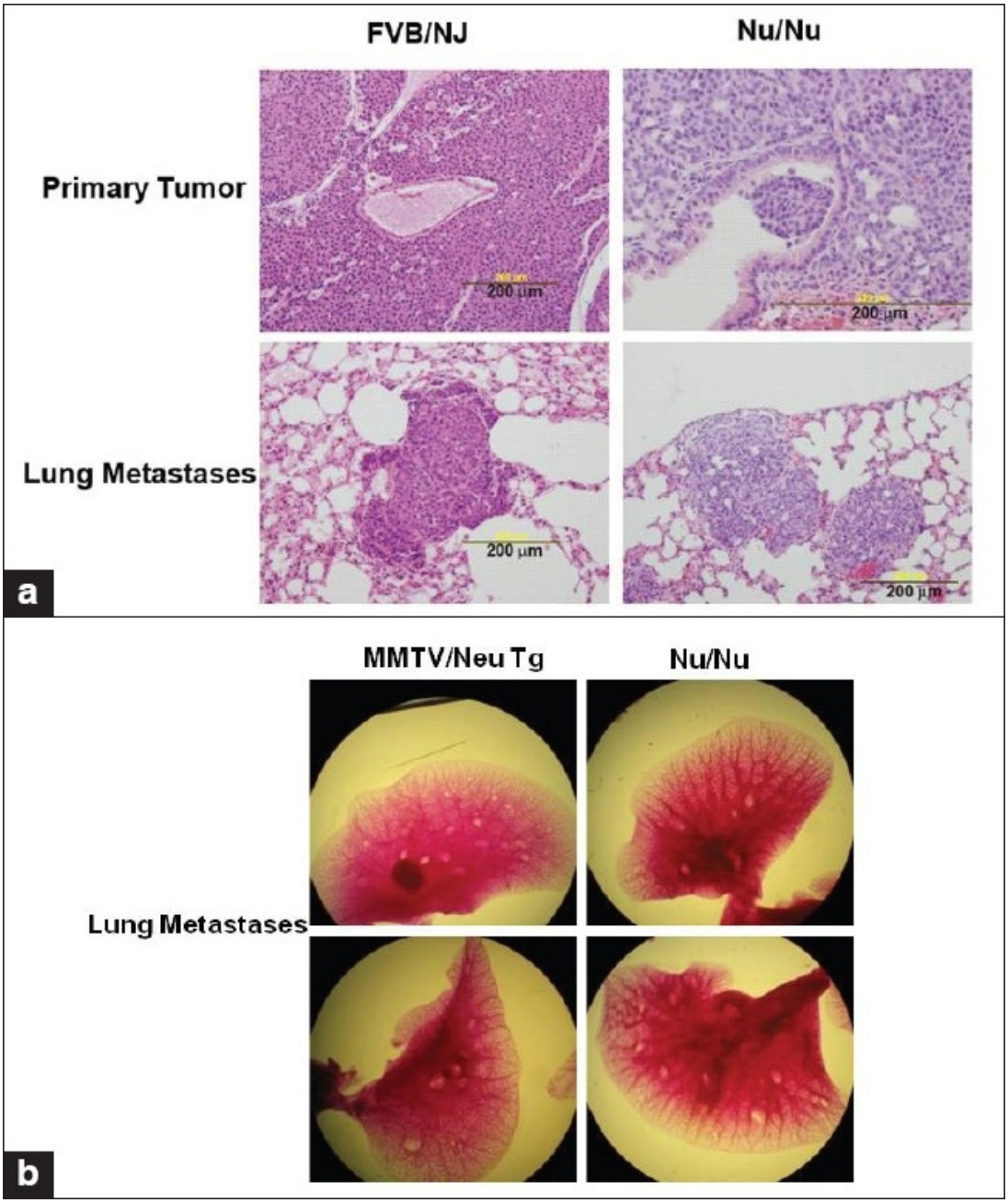


	R1	R2	R3	R4	L
Mouse 1	3	2	0	1	2
Mouse 2	3	3	5	0	1
Mouse 3	3	4	0	3	1
Mouse 4	2	0	1	1	0
Mouse 5	7	4	1	0	0

	R1	R2	R3	R4	L
Mouse 1	2	2	1	6	2
Mouse 2	10	3	6	10	3
Mouse 3	5	2	1	4	1
Mouse 4	5	6	9	1	2
Mouse 5	12	2	7	2	0

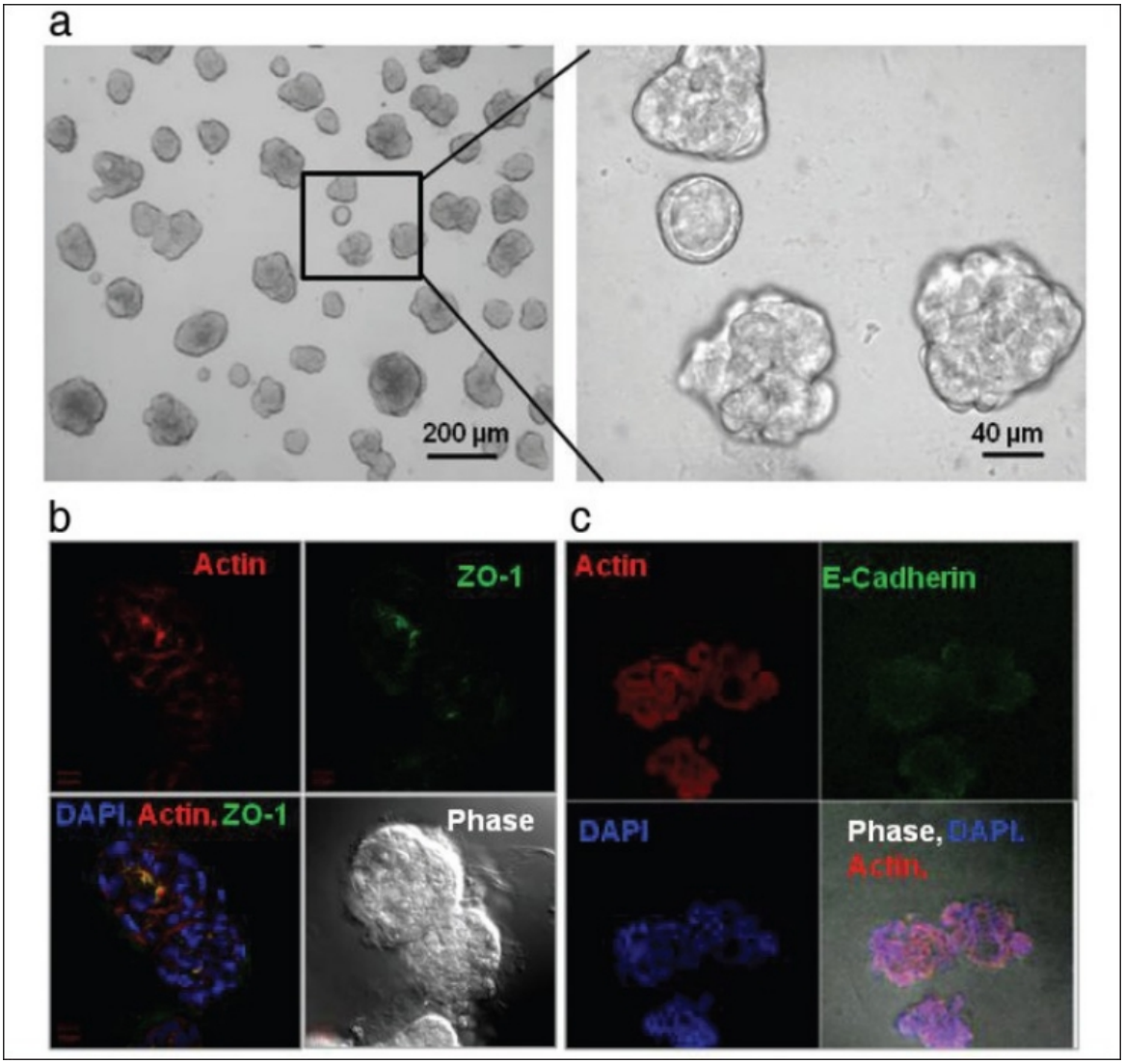
Tumor growth and metastasis of orthotopically implanted MPPS1 cells in MMTV-Neu Tg and Nu/Nu mice: (a) MPPS1 cells were orthotopically implanted into immune-competent ErbB2/Neu transgenic (MMTV-Neu Tg) or immune-compromised Nu/Nu mice (n = 5 per group) and tumor growth monitored as described in the methods section. Shown here is a comparison of tumor growth in MMTV-Neu Tg vs. Nu/Nu background mice. (b) Lungs from the mice used in A were analyzed for metastatic tumor nodules. Shown is a comparison of the numbers of metastatic lesions recorded in various parts of the lung (see illustration in the inset).

Figure 3



Comparative histopathological analysis of primary tumors and lung metastasis of MPPS1 cells orthotopically implanted into mammary fat pads of syngeneic immune-competent mice (FVB/NJ or MMTV-Neu Tg) or immune-compromised Nu/Nu mice: (a) Primary tumors from mammary fat pads and lungs with metastatic lesions developing in FVB/NJ and Nu/Nu mice injected with MPPS1 cells were fixed in formalin and processed for histopathology. Images of primary tumor and lung are from the same animal. Scale bar represents 200 µm. (b) Carmine staining of lungs isolated from MMTV-Neu Tg and Nu/Nu mice that were used in the tumor growth and metastasis study described in the legend to Figure 2. Sites of metastatic lesions are seen as clear areas within carmine-stained lungs. Scale bar represents 1 mm length.

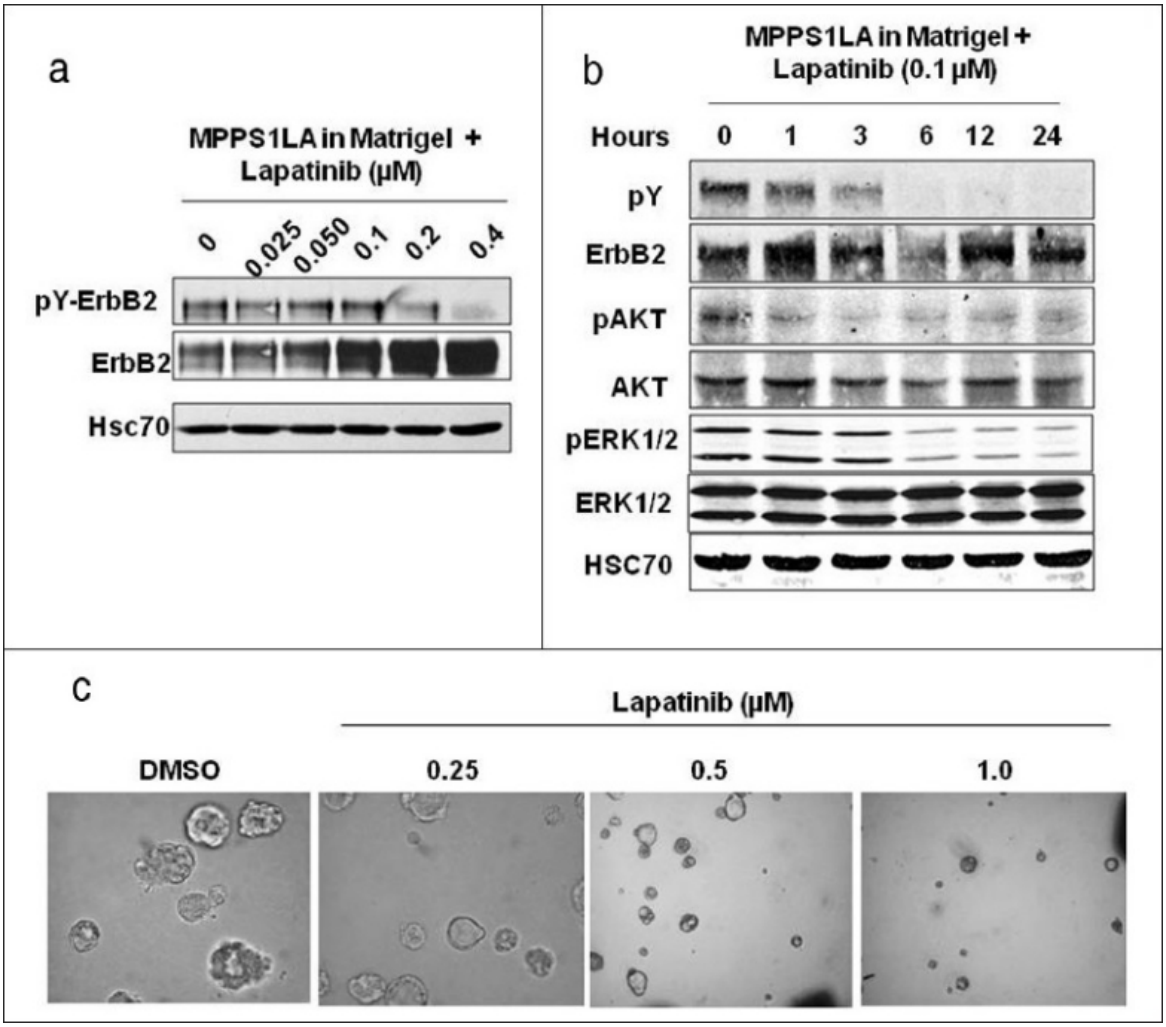
Figure 4



Characterization of the cellular structures formed by MPPS1 cells when grown in 3-D Matrigel culture: (a) Single cell suspensions of MPPS1LA cells were grown on 2% Matrigel as described in the methods section. Growth and morphology of 3-D structures were visualized using phase-contrast microscopy and photographed. Shown here are pictures of 3-D structures at day 8 after seeding. A higher magnification of the boxed area shown in the left panel is depicted in the panel on the right. (b) MPPS1LA cells grown in 2% Matrigel in glass chamber slides for 8 days were fixed and stained with Alexa594-conjugated phalloidin for sub-apical cortical actin, anti-ZO-1 (tight junction marker) using anti-ZO-1 antibody followed by Alexa488-conjugated secondary antibody against anti-ZO-1. The slides were mounted with a medium containing DAPI (to visualize nuclei) and imaged. Shown is a confocal microscopy picture of a 3-D cluster of cells visualizing the organization of actin filaments (red), ZO-1 (green). Also shown is a merged image including the nuclei (blue) and the phase contrast image; (c) MPPS1LA cells similarly grown in 2% Matrigel in glass chamber slides were fixed and stained with Alexa594-conjugated phalloidin or E-Cadherin (basolateral marker) using the anti-E-Cadherin antibodies followed by Alexa488-conjugated secondary antibody (green) against anti-E-Cadherin antibody. The slides were mounted with a medium containing DAPI (to visualize nuclei) and imaged. Images of a Matrigel colony

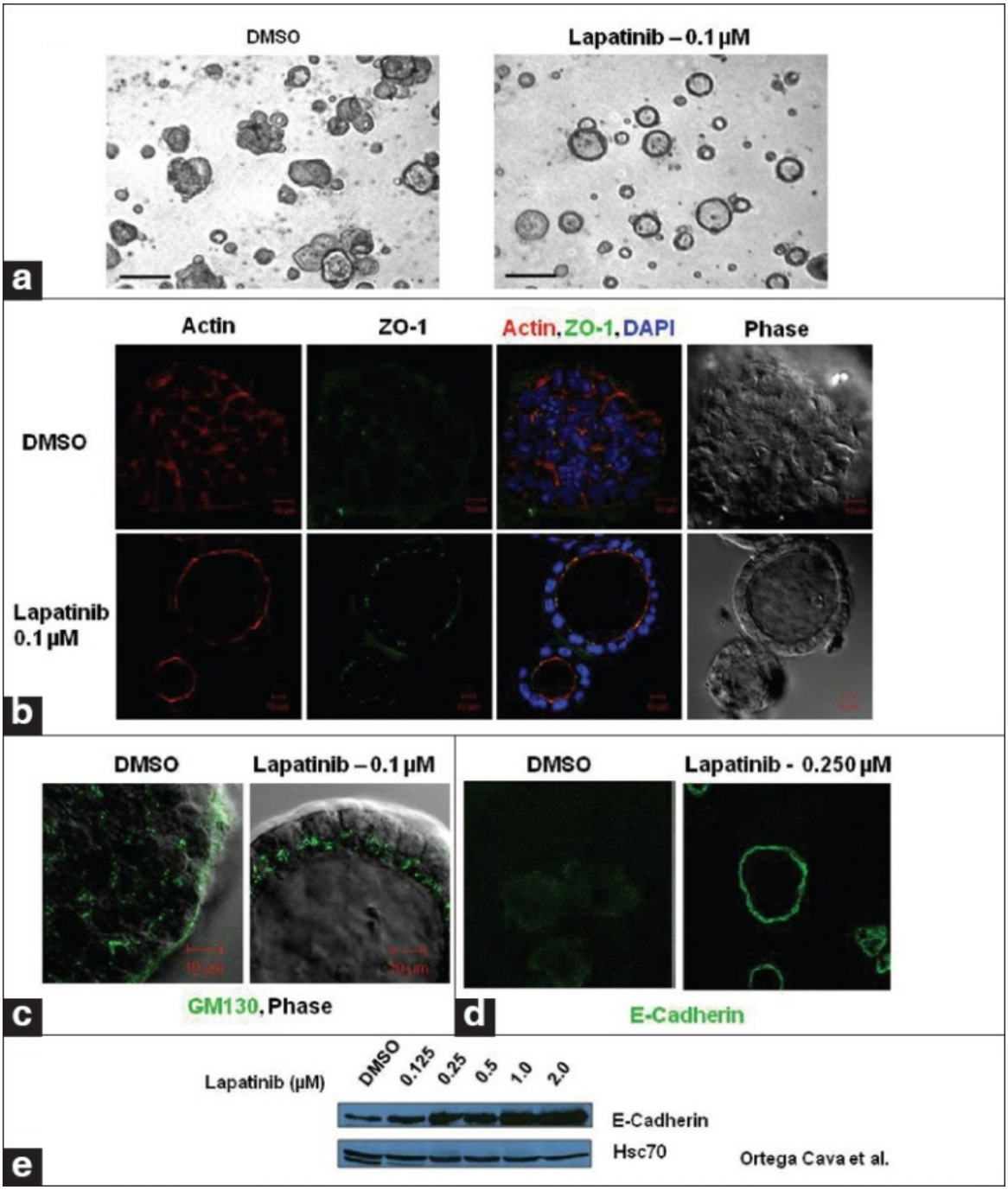
of cells, depicting the disorganized actin filaments (red), and diffuse E-Cadherin (green) distribution. Also shown are the nuclei (in blue) and a merged image showing actin plus E-Cadherin along with the phase contrast.

Figure 5



Analysis of the requirement of ErbB2 kinase activity and downstream signaling pathways in abnormal polarity and associated hyper-proliferation of MPPS1 cells in 3-D culture: (a) Concentration-dependent decrease in pY-ErbB2 in MPPS1LA cells, grown in 3-D Matrigel following treatment with Lapatinib. Cells were treated with the indicated concentrations of Lapatinib for 48 hours. The reduction in pY-ErbB2 levels was assessed by SDS-PAGE/Western blotting analysis from equal amounts of total lysate protein (100 μg). Hsc70 was used as a loading control. (b) Kinetics of decrease in pY-ErbB2 in MPPS1LA cells grown in 3-D. 50 μg total lysate protein was analyzed for each indicated time point. Blot strips were probed first with anti-pY ErbB2, anti-pAKT and anti pERK1/2, and then stripped and reprobed for ERBB2/Neu, AKT and ERK, respectively. (c) MPPS1LA grown on Matrigel for 8 days, were treated with the indicated concentrations of Lapatinib for another 6 days. The changes in colony morphology were recorded as described in the legend to Figure 4a. Representative images are shown to illustrate the concentration-dependent effect of Lapatinib on the morphology and size of 3-D structures.

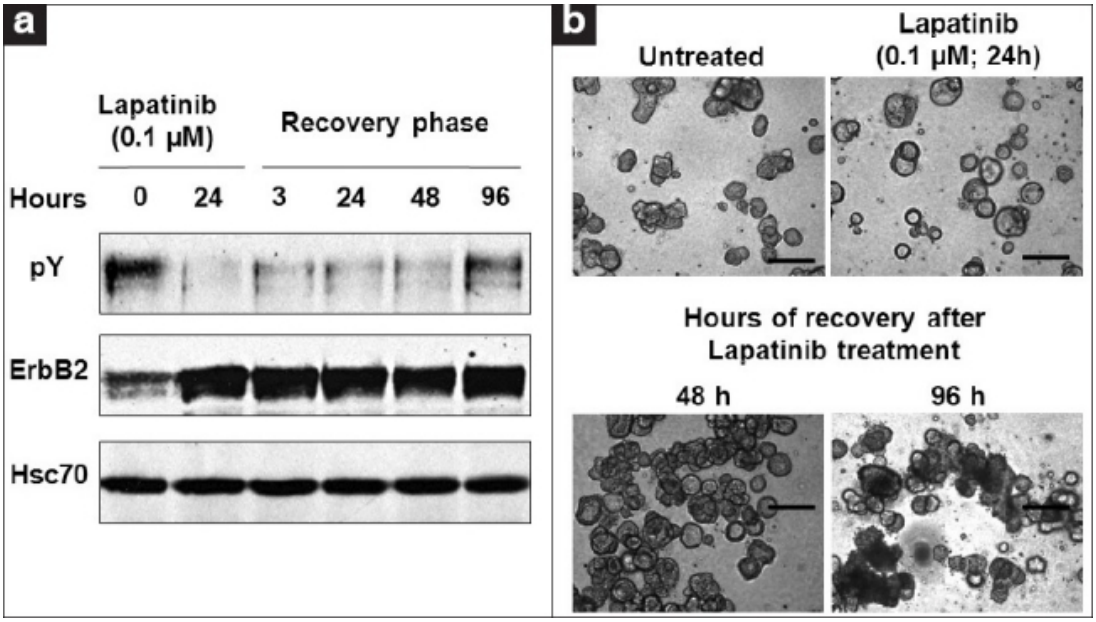
Figure 6



Characterization of Lapatinib-induced normalization of epithelial cell polarity in 3-D Matrigel cultures of MPPS1 cell line. (a) MPPS1 LA cells were grown on Matrigel for 5 days and then were treated with 0.1 μM Lapatinib for 3 days, visualized using phase-contrast microscopy and photographed. Shown is a comparison of the morphology for 3-D structures in cultures treated with DMSO (left panel) or 0.1 μM Lapatinib (right panel)., (b-d) Cells grown on Matrigel (similar to 6A) in 8-well chamber slides were treated with DMSO or 0.1 μM Lapatinib. Following treatment, the cells were paraformaldehyde fixed and stained with anti-ZO1 (B) or anti-GM1 (c; apical marker) or anti-E-cadherin (D) followed by

Alexa488-conjugated secondary antibody and Alexa594-phalloidin (for actin). The slides were mounted with a medium containing DAPI and imaged; (e) Lapatinib treatment-induced accumulation of E-cadherin protein levels in MPPS1LA cells; shown is a western blot analysis of 25 μg total proteins from lysates of MPPS1LA cells treated with the indicated concentrations of Lapatinib for 24h. Hsc70 is shown as a loading control.

Figure 7



Reversal of Lapatinib-induced normalization of epithelial cell polarization in 3-D Matrigel upon removal of inhibitor: MPPS1LA cell grown in 6-well plates on Matrigel were treated with 0.1 μM Lapatinib or DMSO vehicle for the indicated times. For evaluating the reversibility of Lapatinib-induced normalization of epithelial architecture and lumen formation, control and Lapatinib-treated 3-D structures were harvested from Matrigel cultures, spun, washed with PBS and replated in fresh Matrigel. The cultures were imaged at the indicated times. The results are representative of three experiments. a, Western blotting of 50 μg protein confirming the effects of Lapatinib-treatment on pY-ErbB2 and total ErbB2 levels and a time-dependent recovery of pY-ErbB2 following Lapatinib withdrawal. b, Digital images of cell structures, left untreated, Lapatinib treated (24 h), or recovery (48 or 96 h) after Lapatinib withdrawal, are shown. Scale bars are 100 μm.

Journal of Carcinogenesis

Home
Browse articles
Instructions
Submit articles

J Carcinog. 2011; 10: 28. PMCID: PMC3243087
Published online 2011 November 30. doi: 10.4103/1477-3163.90442

Mechanisms of Trastuzumab resistance in ErbB2-driven breast cancer and newer opportunities to overcome therapy resistance

Tameka A. Bailey,[1] Haitao Luan,[1,2] Robert J. Clubb,[1] Mayumi Naramura,[1,2,6] Vimla Band,[1,2,6] Srikumar M. Raja,[1,6,*] and Hamid Band[1,2,3,4,5,6,*]

[1]Eppley Institute for Research in Cancer and Allied Diseases, College of Medicine, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
[2]Department of Genetics, Cell Biology and Anatomy, College of Medicine, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
[3]Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
[4]Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
[5]Department of Pharmacology and Experimental Neuroscience, College of Medicine, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
[6]UNMC-Eppley Cancer Center, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE, USA
Srikumar M. Raja: Sraja@unmc.edu ; Hamid Band: Hband@unmc.edu
[*]Corresponding author

Received September 17, 2011; Accepted October 20, 2011.

Abstract

The Human Epidermal Growth Factor Receptor 2 (Her2, ErbB2 or Neu) is overexpressed in about 20 – 25% of breast cancers and is causally linked to oncogenesis, providing opportunities for targeted therapy. Trastuzumab (Herceptin™, Genentech Inc, San Francisco, CA), a humanized monoclonal antibody against ErbB2, is a successful example of this concept and has vastly improved the response to treatment and overall survival in a majority of ErbB2+ breast cancer patients. However, lack of response in some patients as well as relapse during the course of therapy in others, continue to challenge researchers and clinicians alike towards a better understanding of the fundamental mechanisms of Trastuzumab action and resistance to treatment. The exact *in vivo* mechanism of action of Trastuzumab remains enigmatic, given its direct effects on the ErbB2 signaling pathway as well as indirect contributions from the immune system, by virtue of the ability of Trastuzumab to elicit Antibody-Dependent Cellular Cytotoxicity. Consequently, multiple mechanisms of resistance have been proposed. We present here a comprehensive review of our current understanding of the mechanisms, both of Trastuzumab action and clinical resistance to Trastuzumab-based therapies. We also review newer strategies (based on ErbB2 receptor biology) that are being explored to overcome resistance to Trastuzumab therapy.

Keywords: ErbB2 (Her2/Neu), mechanism, resistance, trastuzumab

INTRODUCTION

ErbB2 (Her2/Neu) is a member of the ErbB family of receptor tyrosine kinases (RTKs), which includes

EGFR (ErbB1, Her1), ErbB3 (Her3), and ErbB4 (Her4). ErbB2 is the preferred dimerization partner for ErbB1, 3, and 4 following growth factor stimulation by ligands such as EGF, TGF-α, and amphiregulin (for EGFR) or Heregulins/Neuregulins (for ErbB3/4).[1] Oncogenic signaling by ErbB2 involves a sustained activation of a number of pathways, including the Ras-Raf-MAPK pathway, which contributes to enhanced cellular proliferation, and the PI3K-Akt pathway, which imparts cell survival among other important biological effects.

Trastuzumab (or Herceptin™ ; manufactured by Roche-Genentech, CA, USA), a humanized monoclonal antibody against the extracellular region of ErbB2, has served as a remarkable example of a successful targeted therapeutic agent in breast cancer. Trastuzumab therapy has significantly contributed to improvements in the treatment outcome of ErbB2-driven breast cancer patients, prolonging their lives. Approximately 20 – 25% of these patients have overexpression of ErbB2, a result in most cases of the amplification of the genomic locus that includes the *ErbB2* gene. Increased levels of the ErbB2 protein can also be a result of altered transcriptional control of *ErbB2* gene expression or of biosynthetic and/or endocytic regulation of cell surface receptor levels. As the likelihood of response to Trastuzumab therapy positively correlates with the ErbB2 protein levels, patient selection typically involves assessment of the ErbB2 status by fluorescent or chromogenic *in situ* hybridization (FISH/CISH) and/or immunohistochemistry (IHC). Clinically, although monotherapy may be effective in some cases, Trastuzumab is invariably given in combination with standard chemotherapy (DNA-damaging drugs, anti-metabolites or microtubule stabilizers). Clinical studies have shown that this combination produces far better response rates than chemotherapy alone and the combinations that include Trastuzumab are now considered as the standard of care for ErbB2-overexpressing breast cancer patients.[2]

Despite the promising initial responses to Trastuzumab therapy in a majority or patients, a subset of patients fails to benefit from treatment, displaying primary or *de novo* resistance. Even within the responders, acquisition of resistance during the course of treatment (secondary resistance) is an additional challenge. Therefore, intense investigations to understand the factors that contribute to the resistance and to identify therapeutic strategies to overcome the resistance are underway at various levels, including cell biological studies, pre-clinical models, and clinical biomarker discovery. However, the effort has faced some fundamental challenges for a number of reasons. First, the exact mechanism of action of Trastuzumab, especially *in vivo*, is unclear. Second, as Trastuzumab given in combination with chemotherapy is the preferred treatment option, it has been difficult to gauge whether the clinical resistance factors are associated with the mechanism of action of Trastuzumab or that of the chemotherapeutics used in combination. Here, we make an attempt to analyze the reported findings in clinical literature, in the context of our current understanding of the mechanism of Trastuzumab as well as the potential mechanisms of its synergy, with the currently used chemotherapeutics. Furthermore, we review newer strategies based on ErbB2 receptor biology that are being explored to overcome clinical resistance associated with Trastuzumab-based therapies.

MECHANISM OF TRASTUZUMAB ACTION

In this section, we will briefly discuss the various mechanisms of action of Trastuzumab that have been proposed, as summarized in Figure 1a.

Attenuation of ErbB2 signaling

Functioning as a monoclonal antibody, the molecular target recognized by Trastuzumab is an antigenic region on the extracellular domain of the ErbB2 receptor. Trastuzumab binding is thought to inhibit the signaling function of ErbB2, resulting in multiple possible mechanisms by which it may exert its anti-proliferative and therapeutic function *in vitro* and *in vivo*. Trastuzumab treatment of breast cancer cells *in vitro* primarily results in the inhibition of proliferation rather than cytotoxicity, which has been linked

to the interruption of PI3K-AKT signaling, resulting in increased nuclear accumulation of the cell cycle inhibitor $p27^{Kip1}$ (as discussed later in the text under section 2.2) and subsequent inhibition of CDK2 activity.[3–5] Mechanistically, this can result from either blockage of the homo- or heterodimer formation or signal attenuation through receptor endocytosis followed by lysosomal degradation. These are indeed two of the proposed mechanisms of action of Trastuzumab.[6,7] Although *in vitro* cell biological and biochemical studies have demonstrated (and we have confirmed) that Trastuzumab treatment can result in ErbB2 internalization and degradation,[8,9] evidence of ErbB2 downregulation *in vivo* (either in pre-clinical xenograft models or clinical studies) is lacking. In a pre-clinical study using BT-474 xenografts and comparing the effect of the anti-EGFR kinase inhibitor, ZD1839 (Gefitinib or Iressa®,AstraZeneca, UK), in combination with Trastuzumab , the authors did not observe any decrease in the ErbB2 levels in tumors harvested after treatment with Trastuzumab alone or its combination with ZD1839.[10] In a clinical study reported by Mohsin *et al*.,[11] involving data on 35 patients treated with Trastuzumab monotherapy in a neoadjuvant setting, no downregulation of ErbB2 was observed.[11] Similarly, contrary to cell biological studies, the expected increase in $p27^{Kip1}$ following Trastuzumab treatment was also not seen in this study. Surprisingly, the authors reported clinical tumor regressions among responders, suggesting that Trastuzumab treatment probably induced apoptosis of tumor cells. As this study involved a limited cohort of patients, expanded studies are needed to verify these findings.

Trastuzumab has also been proposed to function by inhibiting the cleavage of the N-terminal extracellular domain of full length ErbB2 (p185ErbB2), which results in a shorter C-terminal fragment of ErbB2 (described as p95ErbB2 or p95Her2) that can form a hyperactive disulfide-linked homodimer, which initiates oncogenic signaling, but is resistant to Trastuzumab as it lacks the Trastuzumab-binding region.[12] In fact, a phase II clinical study[13] found lower levels of the ErbB2-extracellular domain (ErbB2-ECD) in the serum of patients that responded, consistent with decreased oncogenic signaling, as a result of reduced generation of p95ErbB2. Notably, the truncated forms of ErbB2 can also result from alternate translation initiation sites within the ErbB2 mRNA.[14,15] Regardless of the mechanism of the generation of p95ErbB2, it lacks the binding epitope for Trastuzumab, and therefore, can be an important determinant of Trastuzumab-refractoriness.

Another proposed mechanism of the Trastuzumab-mediated attenuation of ErbB2-PI3K-Akt signaling is through its potential to reactivate the dual phosphatase, Phosphatase and tensin homolog (PTEN), by antagonizing the interaction between ErbB2 and c-Src.[16] In this model, the authors propose that the association of the c-Src non-receptor tyrosine kinase with ErbB2 leads to the phosphorylation of tyrosine residues on the membrane-binding C2 domain of PTEN, resulting in the mislocalization of PTEN and a subsequent increase in the PIP_3 levels, resulting in hyperactive PI3K-Akt signaling.[16] Binding of Trastuzumab is thought to disrupt the interaction of ErbB2 with c-Src, resulting in the reactivation of PTEN, allowing it to translocate to the membrane and attenuate the PIP_3 levels.[16] Conversely, the protein phosphatase activity of PTEN has also been proposed to dephosphorylate c-Src, to regulate its activity.[16,17] Thus, c-Src and PTEN may constitute a complex interdependent factor that ultimately determines Trastuzumab responsiveness through its downstream effect on the PI3K-Akt pathway. Indeed PTEN was subsequently identified, through high-throughput RNA interference screens, to be one of the critical factors associated with Trastuzumab-responsiveness.[18]

The eventual outcome of the attenuation of ErbB2 signaling, by one or more of the mechanism(s) of Trastuzumab action discussed earlier, is the attenuation of PI3K-Akt signaling, which leads to cytostatic effects of Trastuzumab. One of the downstream targets of Akt, is $p27^{kip1}$, which has been implicated in the mechanism of action of Trastuzumab, as discussed herewith.

$p27^{Kip1}$-induced cell cycle arrest

As discussed earlier, interruption of the ErbB2-PI3K-AKT signaling axis, either through a Trastuzumab-

induced block to receptor activation or through receptor internalization and degradation, results in a G1-cell cycle arrest via stabilization of $p27^{kip1}$. Given that one of the downstream consequences of hyperactive ErbB2 signaling is a transcriptional induction of genes involved in DNA repair,[19] it can be hypothesized that Trastuzumab-mediated interruption of ErbB2 signaling can result in an attenuated repair activity following DNA-damaging chemotherapeutics or radiation when Trastuzumab is combined with chemotherapy or radiation; in fact, *in vitro* studies appear to support this hypothesis,[20] and may explain the superior pre-clinical as well as clinical response of Trastuzumab, in combination with Cisplatin or Doxorubicin, in comparison to Trastuzumab monotherapy.[21–24] On the other hand, the combinations of Trastuzumab with Paclitaxel or Docetaxel also exhibit pharmacological synergy *in vitro*, [25,26] and have an even better clinical outcome than with DNA-damaging drugs.[27] However, the mechanism of synergy is unclear and is more difficult to explain by the effect of Trastuzumab on DNA repair, (which follows G1- or G2/M-arrest), as taxols act at the level of microtubules to cause mitotic arrest. ErbB2-overexpression has been associated with the upregulation of Survivin,[28–30] which belongs to the Inhibitors of Apoptosis (IAP) family, but is also an important component of the kinetochore complex in association with INCENP, Aurora B, and Borealin proteins.[31] It is tempting to speculate that the mechanism of synergy may involve the downstream effects of Trastuzumab-mediated disruption of ErbB2 signaling on Survivin function in relation to mitosis.

Even as the therapeutic effect of Trastuzumab may be derived from multiple mechanisms discussed earlier, a common factor appears to be the attenuation of PI3K-Akt signaling and its consequence on cell cycle regulation. Not surprisingly, the multiple mechanisms suggested to be contributory to Trastuzumab refractoriness,[7,32] as will be discussed later in the text, also seem to converge on the PI3K-Akt pathway.

Antibody-Dependent cellular cytotoxicity

As Trastuzumab is an intact monoclonal antibody (IgG1), the Fcγ portion of the molecule can play a significant role in its *in vivo* activity, by its ability to engage the Fcγ receptors on immune effector cells, such as, macrophages, NK cells or cytotoxic T cells, to elicit Antibody-Dependent Cellular Cytotoxicity (ADCC).[33–36] In fact, pre-clinical studies using F(ab′)2 fragments of anti-ErbB2 antibodies[37] or mice deficient in Fcγ receptor activation[38] show severely attenuated anti-tumor responses to Trastuzumab in the xenograft models. Notably, clinical studies in neo-adjuvant settings have revealed increased leukocyte infiltration within the tumor tissue following Trastuzumab treatment.[39] A recent clinical finding that Fcγ receptor polymorphisms may be determinants of Trastuzumab response in breast cancer patients[40] supports the potential role of ADCC in Trastuzumab-based therapies. Tumor regression, reported in clinical studies,[11,41] may also perhaps be explained by ADCC-mediated cytotoxic responses, as opposed to the cytostatic effects of Trastuzumab seen *in vitro*. When combined with chemotherapy, Trastuzumab has been clearly shownto be vastly superior,[24,42] although the exact mechanisms of synergy are unclear. Taken together, this suggests that the ADCC-independent mechanisms are equally important in the anti-tumor activity of Trastuzuamb. However, while ADCC may be a predominant mechanism in Trastuzumab monotherapy, it could have a more limited role when Trastuzumab is given in combination with chemotherapy, given the cytotoxic effects of chemotherapeutics on immune cells. This complexity has not been thoroughly addressed experimentally.

MECHANISMS OF RESISTANCE TO TRASTUZUMAB-BASED THERAPIES

Although clinical resistance to Trastuzumab-based therapies is understood as lack of response to treatment (either *de novo* or acquired during the course of treatment), the proposed mechanisms causing resistance (or refractoriness) come primarily from *in vitro* cell culture studies, in the context of Trastuzumab monotherapy. Potential tumor cell-intrinsic resistance factors include: (1) loss or

inactivation of the PTEN tumor suppressor and subsequent over-activation of the PI3K pathway;[16] (2) mutant PI3K expression;[18] (3) lack of Trastuzumab binding due to expression of p95ErbB2 or steric hindrance to the Trastuzumab-binding site on ErbB2 caused by its cell-surface association with heavily glycosylated proteins such as Muc4 or CD44-hyaluronan;[15,43,44] and (4) amplification/overexpression of Cyclin E.[45] These are summarized in Figure 1b and discussed herewith.

Hyperactivation of PI3K-Akt pathway through PTEN-loss, PI3K mutations, alternative growth factor receptor or p95ErbB2 signaling

During the course of the treatment, several genetic or environmental alterations can accumulate within the tumor or its microenvironment, such as loss/inactivation of the PTEN tumor suppressor, activating PI3K mutations, and dependence on signaling through alternative growth factor receptors including EGFR, p95ErbB2, ErbB3, Insulin-like growth factor receptor (IGF-1R), and other RTKs.[46–48] Alternately, the interaction of tumor cells with the surrounding stroma can lead to reconditioning of the tumor microenvironment, particularly changing the abundance of activating RTK ligands present within the tumor microenvironment. For example, Wang *et al*.,[49] have proposed that TGF-β, present within the tumor microenvironment can lead to increased shedding of ligands, Heregulin, amphiregulin, and TGF-α, via TACE/ADAM17 relocalization to the plasma-membrane may be a contributing factor, leading to Trastuzumab resistance.[49] All these factors can contribute to acquired-resistance to therapy.

The net effect of PTEN-loss/inactivation or expression of mutant PI3K, p95ErbB2 or alternate RTKs is the hyperactivation of the PI3K-Akt signaling pathway. The PI3K-Akt/ PTEN signaling network constitutes a major pathway in the regulation of cell proliferation, metabolism, and anti-apoptotic signal transduction.[50] Hyperactive Akt signaling affects the activity of several of its targets,[51–53] such as, the cell cycle regulator $p27^{kip1}$[54] the pro-apoptotic protein BAD,[55] as well as the FOXO family of transcription factors[56,57] (which regulate the transcription of pro-apoptotic effectors) by phosphorylation. The phosphorylated target proteins remain sequestered from its site of action via binding to 14-3-3 proteins,[54,55,57,58] as is the case with $p27^{kip1}$, where $p27^{kip1}$ is unable to enter the nucleus to inhibit CDK2/CDK4 activity.[58] Similarly, sequestration of phosphorylated-FOXO transcription factors in the cytoplasm by 14-3-3 proteins prevents transcription of its target proteins, including pro-apoptotic effectors such as BNIP3L.[59]

Deregulation of PI3K-Akt signaling or loss of the *PTEN* gene correlates with ErbB2+ tumor progression and maintenance as well as Trastuzumab resistance. Efficacy of Trastuzumab depends not only on the ErbB2 status of breast tumors, but also on aberrations of the genes that encode the PI3K-Akt/PTEN pathway.[16] In particular, retrospective evaluation of formalin-fixed paraffin-embedded tissue samples, isolated from 227 patients with metastatic breast cancer, and treated with Trastuzumab, reveal a predictive role of PIK3CA activating mutations and loss of PTEN in patient responsiveness. The shorter time to progression of metastatic breast cancer in patients correlates with the ErbB2+ status and PIK3CA mutations. However, loss of PTEN results in reduced overall survival irrespective of the ErbB2 status.[60] Interestingly, the PIK3CA activating mutations are mutually exclusive with PTEN deletion, as would be anticipated, as these two proteins catalyze the same reaction in opposite directions, to regulate the PIP_3 levels, and thus, there would be little selective advantage from their concurrent alterations.[61] Mutations of the *PIK3CA* genes occur at frequencies of up to 40% in human breast cancers, although the mutations are not exclusively associated with ErbB2+ breast cancers.[61–64] A majority of the activating mutations of PI3K occur within exons 9 and 20 of the *PI3KCA* gene and encode the central helical domain and C-terminal kinase domain of PI3K, respectively.[61,62,65] Expression of PIK3CA, harboring single amino acid substitutions at E545K or H1047R in the immortalized breast cancer cell line MCF-10A, results in growth factor-independent proliferation and

anchorage-independent growth, as a consequence of the constitutive activation of the kinase and its downstream target, Akt. Furthermore, overexpression of wild-type PIK3CA and its constitutively-active mutants, in two ErbB2 overexpressing cell lines, BT-474 and SKBR3, confer Trastuzumab resistance[18] and abrogate the cytostatic response to Trastuzumab. These studies suggest that the activating mutations of PIK3CA may be one mechanism of *de novo* resistance to Trastuzumab and may perhaps contribute to the lower efficacy of Trastuzumab as a monotherapy. Interestingly, a recent report has suggested that Trastuzumab resistance due to PTEN inactivation can occur via Erythropoeitin Receptor (EpoR)-mediated Src activation. This is a novel mechanism in a Trastuzumab chemotherapy setting, which requires further validations, as recombinant erythropoietin is used to counter erythropenia due to chemotherapy.[66]

Escape from cell-cycle arrest

One consequence of activation of the PI3K-Akt pathway is the G1/S phase cell-cycle progression of human mammary epithelial cells through a mechanism that is partially associated with the change in localization or downregulation of $p27^{Kip1}$.[67] Akt-driven phosphorylation of p27 leads to its translocation from the nucleus to the cytoplasm, thereby, inhibiting its interaction with CDK2/cyclin E1.[53] The consequence of lack of $p27^{kip1}$/CDK2/cyclin E1 complex formation is a release of CDK2/cyclin E1 from inhibition, which induces cell-cycle progression and cell proliferation. Human breast cancer patients that express cytoplasmic $p27^{kip1}$ have reduced rates of survival. In tumors from such patients, the mislocalization of p27 is associated with the activation of Akt.[53,68,69] Interestingly, Trastuzumab or an inhibitor of PI3K can redistribute $p27^{kip1}$ from a cytoplasmic plus nuclear to predominately nuclear distribution in BT-474 cells. However, induction of nuclear translocation of p27 by Trastuzumab does not occur in BT-474 cells lines with acquired resistance to Trastuzumab,[70] thereby indicating that the subcellular location of $p27^{kip1}$ is important for the cytostatic and cytotoxic properties of Trastuzumab and resistance. Secondary to $p27^{kip1}$ redistribution, Trastuzumab also lengthens the half-life of $p27^{kip1}$ by inhibiting CDK2 activation, a prerequisite for $p27^{kip1}$ protein degradation via the ubiquitin proteasome pathway.[70–73] Stabilization of the $p27^{kip1}$ protein allows for complex formation of $p27^{kip1}$/CDK2-Cyclin E, resulting in a decrease in cell proliferation. Notably, reduction of the $p27^{kip1}$ levels by ubiquitin proteasome-dependent degradation results in the elevation of CDK2 activity in the Trastuzumab-resistant SKBR3 cells.[72] These studies suggest that ErbB2 may regulate the functions of CDK2 and Cyclin E, and that both proteins may contribute to Trastuzumab resistance. In support of these observations, the suppression of ErbB2 expression by siRNAs in SKBR3 cells results in the decreased expression of cyclin E and the activities associated with this protein.[74] Moreover, treatment of SKBR3 cells with Trastuzumab results in a decrease in the cyclin E protein levels, relocalization of p27 into the nucleus, as well as inhibition of cell-cycle progression and activation of apoptosis.[74] Furthermore, Trastuzumab-resistant, ErbB2-amplified BT-474 cells overexpress Cyclin E isoforms and exhibit elevated activity associated with the CDK2-Cyclin E complex. Suppression of Cyclin E expression by siRNAs in the Trastuzumab-resistant, ErbB2-amplified BT-474 cell line results in restoration of Trastuzumab sensitivity.

Overexpression of Cyclin E may also contribute to the efficacy of Trastuzumab in the clinic. Specifically, the comparison of ErbB2+ patients with or without overexpression of Cyclin E has revealed that the coexpression of Cyclin E with ErbB2 resulted in shorter time to progression and poorer overall outcome in response to Trastuzumab.[45] In addition, patients with ErbB2+ tumors that overexpress Cyclin E have a worse prognosis in comparison to those that have ErbB2+ tumors with low Cyclin E.[75] However, an evaluation of tissue samples isolated from patients with various stages of cancer has revealed that the expression of cyclin E serves as a prognostic marker, irrespective of the ErbB2 status.[76] Thus, these correlations need to be further explored with in-depth mechanistic analyses.

Evasion of immune-mediated cytotoxic responses

The robustness of an ADCC-mediated response to Trastuzumab therapy is highly dependent on the patient's immune system.[77] Resistance mechanisms, potentially due to altered immune mechanisms include: (1) polymorphisms within Fcγ receptors expressed on immune cells that can affect the affinity for Trastuzumab Fc region binding;[40] (2) increased expression of Killer Inhibitory Receptors (KIRs) on NK cells, which can suppress NK activity;[78] (3) immunosuppression through cytokines produced by tumor cells;[79] and (4) tumor-intrinsic expression of the BH3-family of anti-apoptotic proteins that can antagonize Granzyme B-Perforin-induced apoptosis by cytotoxic lymphocytes/NK cells.[80] Again, an experimental analysis of the role of these mechanisms using appropriate animal models in the context of combined Trastuzumab plus chemotherapy regimens should further shed light on the relative roles of immune versus signaling mechanisms in therapeutic resistance.

Breast Cancer Stem Cells

Tumor Initiating Cells (TICs) or Cancer Stem Cells (CSCs) are thought to contribute to tumor recurrence after adjuvant treatments.[81–84] As the adjuvant therapy of ErbB2-driven breast cancers typically involves a combination of chemotherapy with Trastuzumab, it is important to understand whether the resistance is mechanistically related to the action of Trastuzumab or to the chemotherapeutic being used in the context of TICs/CSCs. Clinical studies have shown that Trastuzumab plus chemotherapy combination in an adjuvant setting has a favorable impact on the relapse rates,[85,86] suggesting that such a regimen may impact TICs/CSCs. Notably, the highest levels of ErbB2 expression have been reported within the TIC sub-population of the ErbB2-overexpressing tumor cell lines.[87,88] In the xenograft models, Trastuzumab treatment has been reported to result in the elimination of TICs/CSCs. This may explain the favorable outcomes reported in the adjuvant settings. Higher ErbB2 expression in TICs / CSCs appears to be at apparent odds with the strong correlation between the levels of ErbB2 overexpression in breast cancers and the Trastuzumab response.[89] It is, however, possible that a higher level of ErbB2 in TICs/CSCs also endures maximal ErbB2 effects in these cells, and ensures that resistance to concurrent chemotherapeutic agents due to escape of TICs/CSCs is minimized. Any potential contribution of TICs to Trastuzumab refractoriness is probably a result of concomitant genetic alterations within the TICs, such as PTEN-loss, PI3K mutations, activation of the NFκB pathway or contributions from the Notch or Wnt signaling pathways, which have been reported to play a role in the maintenance of TICs.[90] To what extent Trastuzumab resistance, whether *de novo* or after therapy, might relate to altered TIC/CSC responses to Trastuzumab needs to be further explored.

NEW THERAPEUTIC OPPORTUNITIES BASED ON THE BIOLOGY OF ERBB2

Several new therapeutic approaches based on insights into ErbB2 receptor biology and better cell biological understanding of the potential resistance mechanisms (as depicted in Figure 1b), are being pursued and are at various stages of pre-clinical or clinical development. These are briefly summarized in Table 1 and some of the more promising approaches are discussed here.

Blocking alternative growth factor receptor signaling

As discussed earlier, a major signaling node in Trastuzumab action appears to be the PI3K-Akt pathway. As signaling downstream of the alternative growth factor receptors implicated in Trastuzumab resistance (such as, EGFR, ErbB3, p95ErbB2, and IGF-1R) converge on this node, several strategies are being tested to block the ability of alternative growth factor receptors to signal. One approach to achieve signal blockage, which has recently achieved considerable success, is the use of monocolonal antibodies that prevent ErbB2 heterodimerization with EGFR or ErbB3. Pertuzumab is one such antibody developed by Genentech (South San Francisco, CA, USA), which blocks the heterodimerization of ErbB2

with other ErbB receptors, especially ErbB3.[91] Pertuzumab in combination with Trastuzumab has been seen to have a higher efficacy in pre-clinical models.[92] Several studies involving Pertuzumab are currently in the Phase II/III trials.[93] Although the results are not published yet, a recent news and analysis report has published a 'Trial Watch' in the Nature Reviews in Drug Discovery (September 2011), announcing the preliminary results from a phase III clinical trial (involving 808 patients) of Pertuzumab plus Trastuzumab in combination with Docetaxel, which has claimed to significantly extend the progression-free survival of patients.[94] Alternatively, downstream signaling from alternative RTKs can be blocked using kinase inhibitors. Lapatinib (Tykerb™, Glaxo SmithKline, UK), a dual EGFR/ErbB2 inhibitor has been recently approved for clinical use in ErbB2+ breast cancer.[95] The addition of Lapatinib, in particular, has been shown to enhance the activity of Trastuzumab in both *in vitro* and *in vivo* studies.[96] Interestingly, the inhibition of ErbB2 kinase activity leads to an increase in the total ErbB2 and ErbB3 levels,[97] which could provide for the increased binding of Trastuzumab and promote anti-tumor effects, as the ErbB2 levels positively correlate with a response to Trastuzumab.[98] The Lapatinib plus Trastuzumab combination is currently in phase II clinical studies [Table 1]. In addition to Lapatinib, which is a reversible inhibitor, several irreversible kinase inhibitors are also being evaluated in cell-based pre-clinical studies as well as in clinical studies.[99] Neratinib (HKI-272) is one such agent that has been reported to be well-tolerated with significant clinical activity in phase II studies. [100] Given the potential role of the heterogeneity of tumors with multiple alternative growth factors contributing to resistance, another approach being explored is the use of multi-targeted kinase inhibitors, such as Sorafenib, in combination with Trastuzumab.[66,101]

Inhibition of downstream PI3K-Akt-mTOR signaling

As increased expression of alternative growth factor receptors, PTEN-loss or mutant PIK3CA expression result in hyperactive PI3K-Akt signaling, inhibitors of PI3K, Akt, and mTOR (downstream target of Akt) as single drugs or in combination with Trastuzumab and/or chemotherapy are also being explored.[102] The PI3K inhibitor XL147 (Exelisis, San Francisco, USA) is currently in phase I/II clinical evaluations, in combination with Paclitaxel (clinicaltrials.gov). Interestingly, a recent study by Chakrabarty *et al*.,[103] has reported that PI3K inhibition leads to upregulation of multiple receptor tyrosine kinases. Such studies should help in the future design of basic research and clinical trials involving rational therapeutic combinations targeted at feedback regulatory pathways, to improve the clinical efficacy of Trastuzumab-based therapies. Targeting of Akt, which is downstream of PI3K, has also been evaluated; however, a phase-2 study did not find any significant objective responses in breast cancer patients with metastatic disease treated with the Akt inhibitor Perifosine (Keryx Biopharmaceuticals, NY, USA).[104] On the other hand, inhibition of mTOR (downstream target of Akt) using RAD001 (Novartis, NY, USA) has been shown to improve the response to Trastuzumab in the breast cancer models of PTEN-loss and provide some clinical benefit in a phase I/II study, involving a small number of patients.[105]

Enhancing Trastuzumab efficacy by targeting HSP90

ErbB2 as well as a number of its downstream signaling proteins, including many implicated in Trastuzumab resistance, such as, ErbB2, p95ErbB2, Akt, CDK2, and cyclin E, are client proteins of the molecular chaperone HSP90. The kinase domains of both full-length ErbB2 and p95ErbB2, form complexes with HSP90 in cultured breast cancer cells and tumors.[106–108] The association of ErbB2 with HSP90 is necessary for the stability and activity of the nascent and mature ErbB2 protein. [9,106,107] Similar to ErbB2, the stability and activation of Akt are dependent on its interaction with HSP90.[109,110] In the case for Cdk2, only the folding and maturation of the protein is probably dependent on HSP90, as only the newly-synthesized protein associates with the chaperone[111] Furthermore, an indirect, novel mechanism for HSP90 chaperoning for Cyclin E, may exist, as it does not directly bind HSP90, but is unstable in the presence of inhibitors of HSP90.[112]

Inhibition of the chaperone function of HSP90 using inhibitors of its ATPase activity such as geldanamycin and its derivative 17AAG results in the ubiquitination and subsequent degradation of client proteins including ErbB2, p95ErbB2, Akt, and cyclin E. Specifically, ErbB2 is rapidly endocytosed from the plasma membrane and/or rerouted from its recycling pathway to the lysosomes, in response to HSP90 inhibition.[9,113,114] The downregulation of ErbB2 correlates with a decrease of ErbB2 signal transduction, including the inactivation of the PI3K-Akt pathway, degradation of cell cycle proteins, inhibition of cell progression, and the induction of apoptosis.[9,115,116] Although the treatment of breast cancer cells with inhibitors of HSP90 or Trastuzumab is sufficient to induce the degradation of ErbB2, a combination of drugs is more effective than either alone, and is associated with a more profound inhibition of ErbB2 signaling.[9,115] Furthermore, this synergy also seems adequate to overcome the resistance to Trastuzumab. For example, evaluation of the xenograft models of Trastuzumab-resistance driven by ErbB2 alone or ErbB2 and p95ErbB2 has shown that a combination of Trastuzumab and HSP90 inhibitors results in a greater decrease of tumor growth than Trastuzumab or the inhibitor alone.[117,118] Clinically, the combination of HSP90 inhibitor Tanespimycin and Trastuzumab was also shown to act synergistically on Trastuzumab-resistant tumors.[118–120] A recent phase II clinical trial of patients with metastatic breast cancer has revealed that a majority of patients experiencing disease progression on initial Trastuzumab therapy, exhibited a partial response or stabilization of the disease when treated with a combination of Trastuzumab and Tanespimycin.[119] These studies suggest that combinatorial treatment of Trastuzumab-refractory metastatic breast cancer patients with Trastuzumab and HSP90 inhibitors can considerably improve the survival of patients with ErbB2+ breast cancer.[118,119,121]

Exploiting overexpressed ErbB2 as an address for the targeted delivery of cytotoxic drugs

Although multiple mechanisms may contribute to Trastuzumab-resistance, it does not appear to be due to loss of ErbB2 overexpression, based on most published cell-line models (as discussed in section 5.1). Therefore, one alternative strategy to overcome Trastuzumab-refractoriness is to exploit the cell-surface overexpression of ErbB2 and the high affinity with which Trastuzumab interacts with the receptor to achieve targeted delivery of cytotoxic drugs conjugated to Trastuzumab. Trastuzumab-MCC-DM1 (T-DM1; DM1 is an anti-mitotic drug based on the Vinca alkaloid Maytansine) is an example of a successful antibody-drug conjugate, based on the rationale of using ErbB2 as an address for the specific delivery of cytotoxic drugs. T-DM1 (Genentech) has demonstrated potent and ErbB2-selective anti-cancer activity in several ErbB2-overexpressing and Trastuzumab-resistant cell-line models.[122,123] T-DM1 has also recently completed phase I and II clinical studies,[124,125] and has been found to be well-tolerated with significant objective response rates and improvements in the progression-free survival of patients. This concept of ErbB2-targeted delivery of cytotoxic drugs is also being explored in conjunction with the nano-particulate drug delivery systems, which utilizes polymeric micelles or liposomes encapsulating conventional chemotherapeutics that are decorated with anti-ErbB2 antibodies, to achieve targeted delivery of the chemotherapeutic payload. These studies are currently in the early stages of development at the cell biology and pre-clinical levels.

Targeting angiogenesis and activating immune effectors

Several additional pathways being explored as a target for overcoming Trastuzumab refractoriness are angiogenesis inhibition using anti-VEGF-A antibody Bevacizumab (Avastin™, Genentech, San Francisco, USA), and by boosting the immune component of Trastuzumab action, using trifunctional antibodies such as Ertumaxomab (Rexomun™, Fresenius Biotech GmbH, Germany).

ErbB2-signaling has been implicated in tumor angiogenesis through the production of VEGF-A; [121,126] therefore, the inclusion of anti-VEGF-A antibody Bevacizumab has been evaluated in

combination with Trastuzumab, Carboplatin, and Paclitaxel. However, the inclusion of Bevacizumab did not seem to provide significant clinical benefits.[127] In fact, in an adjuvant setting, the Bevacizumab plus Trastuzumab combination was not well-tolerated, and was casually linked to Bevacizumab-related toxicities.[127] Early studies with the trifunctional antibody Ertumaxomab (that targets ErbB2, CD3, and the activating Fcγ receptor) have shown promising activity, even in ErbB2 low-expressing cell lines.[128] The phase I clinical studies have reported that the antibody is safe and well-tolerated.[129]

LIMITATIONS, CHALLENGES, AND FUTURE PERSPECTIVES

Models of Trastuzumab-resistance

Despite the wealth of information, a global understanding of the mechanism of resistance to Trastuzumab-based therapies remains unclear. This is partly because of extremely limited cellular and animal models of clinical resistance available for study. Most reported studies have relied on a few cell-line models, which include: (1) BT-474 or SKBr-3 cell lines that have been selected for *in vitro* resistance by continuous culture in Trastuzumab;[70,72,118] (2) resistant clones derived from serially transplanted BT-474 xenografts in immune-compromised mice, continually treated with Trastuzumab *in vivo*;[45] (3) JIMT-1 cell line, which has been established from a patient resistant to Trastuzumab therapy.[130] The first two examples serve as the closest models for acquired resistance. However, as the tumor microenvironment, along with an intact immune system, is thought to play a major role in the cellular reprogramming that leads to resistance, the first two models may not accurately reproduce the characteristics of true acquired resistance. On the other hand, although JIMT-1, the only cell line available as a model of *de novo* resistance, has ErbB2 gene-amplification, the ErbB2 protein levels are much lower than the well-established ErbB2-overexpressing cell lines.[130,131] Moreover, its dependence on ErbB2 for growth is unclear, given the relative differences in sensitivities for growth inhibition by Lapatinib.[131] The cell line has been reported to also carry a mutant *PI3K* gene, have low expression of PTEN and also expresses high levels of Neuregulin 1 (NRG1; a ligand for ErbB3). A systematic endeavor to establish newer cell line models representing both *de novo* as well as acquired resistance from patients as well as direct transplants of patient tumor tissue, as xenotransplants in mice, together with a more thorough characterization of transgenic models, with an intact immune system, should help increase our understanding of Trastuzumab resistance.

Newer genetically defined cell-line models can also be generated based on the identification of specific resistance factors. For example, as many studies appear to confirm PTEN loss as one of the factors, interrogation of the mechanistic role of PTEN in cell-line and experimental animal models, with stable or conditional knockdown/knockout of the *PTEN* gene will be very useful. Similarly as more information becomes available through the genomic analyses of patients who are responsive or resistant to Trastuzumab, newer models should become available. Such information could also hopefully lead to the future development of mouse models of Trastuzumab-resistance, which are currently lacking. These directions should help accelerate efforts to find solutions to overcome therapy resistance in the treatment of ErbB2-driven breast cancers.

Identification of new factors that mediate Trastuzumab-Resistance using high-throughput screening approaches

The phosphatase and tensin homolog (PTEN) is the only factor so far identified using a high-throughput RNAi screen done on the Trastuzumab-sensitive BT-474 cell line that has a clinical correlation as a biomarker for Trastuzumab resistance. Of late, a limited siRNA library (covering human kinases and phosphatases) has been used to screen for additional Trastuzumab resistance factors.[132] The investigators identified additional factors that may be mediators of Trastuzumab resistance, including p27 phosphatase (PPM1H) and PTPN11 and three kinases (DYRK1A, STK10, and STYK1). The meager

number of genes identified in the screens done to date suggests a potential limitation of loss of function strategies alone, as most Trastuzumab-resistance factors identified in other studies represent a gain of function (overexpression or mutation of genes that function as accessories or downstream components of ErbB2 signaling). Use of Trastuzumab-sensitive cell systems in loss of function approaches may therefore provide only part of the answer. Similar approaches on Trastuzumab-resistant cell lines are likely to lead to identification of a wider range of resistance factors. Conversely, the genome-wide overexpression of genes using human Open Reading Frame (ORF) libraries in Trastuzumab-sensitive cell line models could lead to the identification of other factors. A cross-validation of hits from these independent approaches in laboratory and pre-clinical models, in conjunction with clinical assessments of the hits as the potential biomarkers of resistance, would greatly aid in identifying newer therapeutic combinations for overcoming Trastuzumab-resistance.

CONCLUSIONS

Despite economically challenging times, years of public and private non-profit investment in basic and translational cancer research has produced a wealth of knowledge and information about the mechanisms that drive and sustain oncogenic growth in cancer cells, placing us in a good position to rapidly find solutions to treatment challenges. Specifically, our understandings of the mechanism(s) of Trastuzumab as well as pathways that contribute to resistance have significantly improved, since its approval in 1998, as reviewed here. However significant challenges continue to remain in translating these findings toward improving patient outcomes, which will require integration of the efforts of basic scientists, clinicians, and the pharmaceutical industry alike, through active collaboration. Such improvements are likely to come as future studies integrate the molecular, biochemical, and cell biological understanding of the mechanisms of Trastuzumab action and resistance, gleaned from laboratory studies, together with information from the clinical evaluation of potential resistance factors and biomarkers of the Trastuzumab response.

AUTHOR'S PFOFILE

Prof. Hamid Band, Eppley Institute for Research in Cancer and Allied Diseases; Departments of Genetics, Cell Biology and Anatomy, Biochemistry and Molecular Biology, Pathology and Microbiology, and Pharmacology and Experimental Neuroscience, College of Medicine; and UNMC-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE

Dr. Srikumar M Raja, Eppley Institute for Research in Cancer and Allied Diseases, UNMC-Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE

ACKNOWLEDGMENTS

The studies from the author's laboratories were supported by the NIH grants CA87986, CA99163, CA105489, and CA116552, to HB, and CA96844 and CA81076 to VB; Department of Defense Breast Cancer Research Grants W81XWH-11-1-0166 (HB), W81XWH-07-1-0351, and W81XWH-11-1-0171 (V.B), and W81XWH-10-1-0740 (M.N.); the National Cancer Institute (NCI 5U01CA151806-02) (HB and SMR); Nebraska Department of Health and Human Services (LB-506 grant to SMR); and Nebraska Center for Nanomedicine-Center for Biomedical Research Excellence (NCN-COBRE; seed grant to SMR). TAB was a trainee under an NCI Cancer Biology Training Grant (T32 CA 009476) and is currently a Susan G. Komen Foundation post-doctoral Fellow (KG091363). We thank the members of the Band laboratories for their helpful suggestions and discussions. We also thank Mr. Scott Nadeu Jr. for the help with proofreading.

REFERENCES

1. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16:1647–55. [PMCID: PMC1169769] [PubMed: 9130710]

2. Hudis CA. Trastuzumab--Mechanism of action and use in clinical practice. N Engl J Med. 2007;357:39–51. [PubMed: 17611206]

3. Lane HA, Motoyama AB, Beuvink I, Hynes NE. Modulation of p27 / Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. Ann Oncol. 2001;12(Suppl 1):S21–2. [PubMed: 11521716]

4. Le XF, Claret FX, Lammayot A, Tian L, Deshpande D, LaPushin R, et al. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. J Biol Chem. 2003;278:23441–50. [PubMed: 12700233]

5. Le XF, Pruefer F, Bast RC., Jr HER2-targeting antibodies modulate the cyclin-dependent kinase inhibitor p27Kip1 via multiple signaling pathways. Cell Cycle. 2005;4:87–95. [PubMed: 15611642]

6. Austin CD, De Maziere AM, Pisacane PI, van Dijk SM, Eigenbrot C, Sliwkowski MX, et al. Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. Mol Biol Cell. 2004;15:5268–82. [PMCID: PMC532009] [PubMed: 15385631]

7. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: Understanding resistance to her2-targeted therapy in human breast cancer. Nat Clin Pract Oncol. 2006;3:269–80. [PubMed: 16683005]

8. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185HER2 monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol. 1989;9:1165–72. [PMCID: PMC362707] [PubMed: 2566907]

9. Raja SM, Clubb RJ, Bhattacharyya M, Dimri M, Cheng H, Pan W, et al. A combination of Trastuzumab and 17-AAG induces enhanced ubiquitinylation and lysosomal pathway-dependent ErbB2 degradation and cytotoxicity in ErbB2-overexpressing breast cancer cells. Cancer Biol Ther. 2008;7:1630–40. [PMCID: PMC2727620] [PubMed: 18769124]

10. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2 / neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. Cancer Res. 2001;61:8887–95. [PubMed: 11751413]

11. Mohsin SK, Weiss HL, Gutierrez MC, Chamness GC, Schiff R, Digiovanna MP, et al. Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. J Clin Oncol. 2005;23:2460–8. [PubMed: 15710948]

12. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. Cancer Res. 2001;61:4744–9. [PubMed: 11406546]

13. Esteva FJ, Valero V, Booser D, Guerra LT, Murray JL, Pusztai L, et al. Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20:1800–8. [PubMed: 11919237]

14. Pedersen K, Angelini PD, Laos S, Bach-Faig A, Cunningham MP, Ferrer-Ramon C, et al. A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. Mol Cell Biol. 2009;29:3319–31. [PMCID: PMC2698729] [PubMed: 19364815]

15. Arribas J, Baselga J, Pedersen K, Parra-Palau JL. p95HER2 and breast cancer. Cancer Res. 2011;71:1515–9. [PubMed: 21343397]

16. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004;6:117–27. [PubMed: 15324695]

17. Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, et al. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat Med. 2011;17:461–9. [PubMed: 21399647]

18. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12:395–402. [PubMed: 17936563]

19. Le XF, Lammayot A, Gold D, Lu Y, Mao W, Chang T, et al. Genes affecting the cell cycle, growth, maintenance, and drug sensitivity are preferentially regulated by anti-HER2 antibody through phosphatidylinositol 3-kinase-AKT signaling. J Biol Chem. 2005;280:2092–104. [PubMed: 15504738]

20. Boone JJ, Bhosle J, Tilby MJ, Hartley JA, Hochhauser D. Involvement of the HER2 pathway in repair of DNA damage produced by chemotherapeutic agents. Mol Cancer Ther. 2009;8:3015–23. [PubMed: 19887555]

21. Pietras RJ, Fendly BM, Chazin VR, Pegram MD, Howell SB, Slamon DJ. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene. 1994;9:1829–38. [PubMed: 7911565]

22. Amadori D, Milandri C, Comella G, Saracchini S, Salvagni S, Barone C, et al. A phase I/II trial of non-pegylated liposomal doxorubicin, docetaxel and trastuzumab as first-line treatment in HER-2-positive locally advanced or metastatic breast cancer. Eur J Cancer. 2011;47:2091–8. [PubMed: 21665463]

23. Pietras RJ, Pegram MD, Finn RS, Maneval DA, Slamon DJ. Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. Oncogene. 1998;17:2235–49. [PubMed: 9811454]

24. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353:1659–72. [PubMed: 16236737]

25. Krug LM, Miller VA, Patel J, Crapanzano J, Azzoli CG, Gomez J, et al. Randomized phase II study of weekly docetaxel plus trastuzumab versus weekly paclitaxel plus trastuzumab in patients with previously untreated advanced nonsmall cell lung carcinoma. Cancer. 2005;104:2149–55. [PubMed: 16208701]

26. Fujimoto-Ouchi K, Sekiguchi F, Yasuno H, Moriya Y, Mori K, Tanaka Y. Antitumor activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models. Cancer Chemother Pharmacol. 2007;59:795–805. [PubMed: 17031648]

27. Pegram MD, Slamon DJ. Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: Evidence for receptor-enhanced chemosensitivity. Semin Oncol. 1999;26(Suppl 4):89–95. [PubMed: 10482199]

28. Lu J, Tan M, Huang WC, Li P, Guo H, Tseng LM, et al. Mitotic deregulation by survivin in ErbB2-overexpressing breast cancer cells contributes to Taxol resistance. Clin Cancer Res. 2009;15:1326–34. [PMCID: PMC2663973] [PubMed: 19228734]

29. Xia W, Bisi J, Strum J, Liu L, Carrick K, Graham KM, et al. Regulation of survivin by ErbB2 signaling: Therapeutic implications for ErbB2-overexpressing breast cancers. Cancer Res. 2006;66:1640–7. [PubMed: 16452223]

30. Guha M, Altieri DC. Survivin as a global target of intrinsic tumor suppression networks. Cell Cycle. 2009;8:2708–10. [PMCID: PMC2819076] [PubMed: 19717980]

31. Jeyaprakash AA, Klein UR, Lindner D, Ebert J, Nigg EA, Conti E. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell. 2007;131:271–85. [PubMed: 17956729]

32. Baselga J. Treatment of HER2-overexpressing breast cancer. Ann Oncol. 2010;21(Suppl 7):vii36–40. [PubMed: 20943641]

33. Ludwig DL, Pereira DS, Zhu Z, Hicklin DJ, Bohlen P. Monoclonal antibody therapeutics and apoptosis. Oncogene. 2003;22:9097–106. [PubMed: 14663488]

34. Capietto A, Martinet L, Fournié J. Stimulated γδ T cells increase the *in vivo* efficacy of trastuzumab in HER-2+ breast cancer. J Immunol. 2011;187:1031–8. [PubMed: 21670311]

35. Yamaguchi Y, Hironaka K, Okawaki M, Okita R, Matsuura K, Ohshita A, et al. HER2-specific cytotoxic activity of lymphokine-activated killer cells in the presence of trastuzumab. Anticancer Res. 2005;25:827–32. [PubMed: 15868915]

36. Cooley S, Burns LJ, Repka T, Miller JS. Natural killer cell cytotoxicity of breast cancer targets is enhanced by two distinct mechanisms of antibody-dependent cellular cytotoxicity against LFA-3 and HER2/neu. Exp Hematol. 1999;27:1533–41. [PubMed: 10517495]

37. Spiridon CI, Guinn S, Vitetta ES. A comparison of the *in vitro* and *in vivo* activities of IgG and F(ab') 2 fragments of a mixture of three monoclonal anti-Her-2 antibodies. Clin Cancer Res. 2004;10:3542–51. [PubMed: 15161714]

38. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. Nat Med. 2000;6:443–6. [PubMed: 10742152]

39. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell. 2010;18:160–70. [PMCID: PMC2923645] [PubMed: 20708157]

40. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, Missale G, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J Clin Oncol. 2008;26:1789–96. [PubMed: 18347005]

41. Varchetta S, Gibelli N, Oliviero B, Nardini E, Gennari R, Gatti G, et al. Elements related to heterogeneity of antibody-dependent cell cytotoxicity in patients under trastuzumab therapy for primary operable breast cancer overexpressing Her2. Cancer Res. 2007;67:11991–9. [PubMed: 18089830]

42. Ligibel JA, Winer EP. Trastuzumab/chemotherapy combinations in metastatic breast cancer. Semin Oncol. 2002;29(Suppl 3):38–43. [PubMed: 12138396]

43. Nagy P, Friedlander E, Tanner M, Kapanen AI, Carraway KL, Isola J, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res. 2005;65:473–82. [PubMed: 15695389]

44. Palyi-Krekk Z, Barok M, Isola J, Tammi M, Szollosi J, Nagy P. Hyaluronan-induced masking of ErbB2 and CD44-enhanced trastuzumab internalisation in trastuzumab resistant breast cancer. Eur J

Cancer. 2007;43:2423–33. [PubMed: 17911008]

45. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, et al. Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. Proc Natl Acad Sci U S A. 2011;108:3761–6. [PMCID: PMC3048107] [PubMed: 21321214]

46. Nahta R, Esteva FJ. HER2 therapy: Molecular mechanisms of trastuzumab resistance. Breast Cancer Res. 2006;8:215. [PMCID: PMC1797036] [PubMed: 17096862]

47. Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. Cancer Res. 2010;70:299–308. [PubMed: 20028874]

48. Shattuck DL, Miller JK, Carraway KL, 3rd, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res. 2008;68:1471–7. [PubMed: 18316611]

49. Wang SE, Xiang B, Guix M, Olivares MG, Parker J, Chung CH, et al. Transforming growth factor beta engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab. Mol Cell Biol. 2008;28:5605–20. [PMCID: PMC2546920] [PubMed: 18625725]

50. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov. 2005;4:988–1004. [PubMed: 16341064]

51. Wu Y, Shang X, Sarkissyan M, Slamon D, Vadgama JV. FOXO1A is a target for HER2-overexpressing breast tumors. Cancer Res. 2010;70:5475–85. [PMCID: PMC2896450] [PubMed: 20551062]

52. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231–41. [PubMed: 9346240]

53. Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, et al. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med. 2002;8:1153–60. [PubMed: 12244302]

54. Fujita N, Sato S, Katayama K, Tsuruo T. Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. J Biol Chem. 2002;277:28706–13. [PubMed: 12042314]

55. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell. 2005;8:287–97. [PMCID: PMC3203692] [PubMed: 16226704]

56. Burgering BM, Medema RH. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol. 2003;73:689–701. [PubMed: 12773501]

57. Tzivion G, Dobson M, Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta. 2011;1813:1938–45. [PubMed: 21708191]

58. Yang H, Zhang Y, Zhao R, Wen YY, Fournier K, Wu HB, et al. Negative cell cycle regulator 14-3-3sigma stabilizes p27 Kip1 by inhibiting the activity of PKB / Akt. Oncogene. 2006;25:4585–94. [PubMed: 16532026]

59. Real PJ, Benito A, Cuevas J, Berciano MT, de Juan A, Coffer P, et al. Blockade of epidermal growth factor receptors chemosensitizes breast cancer cells through up-regulation of Bnip3L. Cancer Res. 2005;65:8151–7. [PubMed: 16166289]

60. Razis E, Bobos M, Kotoula V, Eleftheraki AG, Kalofonos HP, Pavlakis K, et al. Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic

breast cancer. Breast Cancer Res Treat. 2011;128:447–56. [PubMed: 21594665]

61. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res. 2005;65:2554–9. [PubMed: 15805248]

62. Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther. 2004;3:772–5. [PubMed: 15254419]

63. Campbell CI, Petrik JJ, Moorehead RA. ErbB2 enhances mammary tumorigenesis, oncogene-independent recurrence and metastasis in a model of IGF-IR-mediated mammary tumorigenesis. Mol Cancer. 2010;9:235. [PMCID: PMC2940847] [PubMed: 20825649]

64. Wu G, Mambo E, Guo Z, Hu S, Huang X, Gollin SM, et al. Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors. J Clin Endocrinol Metab. 2005;90:4688–93. [PubMed: 15928251]

65. Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, Pearline RV, et al. Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res. 2005;65:10992–1000. [PubMed: 16322248]

66. Liang K, Esteva FJ, Albarracin C, Stemke-Hale K, Lu Y, Bianchini G, et al. Recombinant human erythropoietin antagonizes trastuzumab treatment of breast cancer cells via Jak2-mediated Src activation and PTEN inactivation. Cancer Cell. 2010;18:423–35. [PMCID: PMC3022383] [PubMed: 21075308]

67. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003;2:339–45. [PubMed: 12851486]

68. Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, et al. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med. 2002;8:1145–52. [PubMed: 12244301]

69. Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, et al. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB / Akt-mediated phosphorylation in breast cancer. Nat Med. 2002;8:1136–44. [PubMed: 12244303]

70. Kute T, Lack CM, Willingham M, Bishwokama B, Williams H, Barrett K, et al. Development of Herceptin resistance in breast cancer cells. Cytometry A. 2004;57:86–93. [PubMed: 14750129]

71. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: Receptor overexpression does not determine growth dependency. Mol Cell Biol. 2000;20:3210–23. [PMCID: PMC85615] [PubMed: 10757805]

72. Nahta R, Takahashi T, Ueno NT, Hung MC, Esteva FJ. P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. Cancer Res. 2004;64:3981–6. [PubMed: 15173011]

73. Le XF, Claret FX, Lammayot A, Tian L, Deshpande D, LaPushin R, et al. The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. J Biol Chem. 2003;278:23441–50. [PubMed: 12700233]

74. Mittendorf EA, Liu Y, Tucker SL, McKenzie T, Qiao N, Akli S, et al. A novel interaction between HER2/neu and cyclin E in breast cancer. Oncogene. 2010;29:3896–907. [PMCID: PMC2900397] [PubMed: 20453888]

75. Potemski P, Pluciennik E, Bednarek AK, Kusinska R, Jesionek-Kupnicka D, Pasz-Walczak G, et al. Cyclin E expression in operable breast cancer quantified using real-time RT-PCR: A comparative study with immunostaining. Jpn J Clin Oncol. 2006;36:142–9. [PubMed: 16520356]

76. Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, et al. Cyclin E and survival in patients with breast cancer. N Engl J Med. 2002;347:1566–75. [PubMed: 12432043]

77. Tagliabue E, Campiglio M, Pupa SM, Menard S, Balsari A. Activity and resistance of trastuzumab according to different clinical settings. Cancer Treat Rev. 2011 ***[In press]***

78. Stein MN, Shin J, Gudzowaty O, Bernstein AM, Liu JM. Antibody-dependent cell cytotoxicity to breast cancer targets despite inhibitory KIR signaling. Anticancer Res. 2006;26:1759–63. [PubMed: 16827104]

79. Trotta R, Col JD, Yu J, Ciarlariello D, Thomas B, Zhang X, et al. TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. J Immunol. 2008;181:3784–92. [PMCID: PMC2924753] [PubMed: 18768831]

80. Kawaguchi Y, Kono K, Mizukami Y, Mimura K, Fujii H. Mechanisms of escape from trastuzumab-mediated ADCC in esophageal squamous cell carcinoma: Relation to susceptibility to perforin-granzyme. Anticancer Res. 2009;29:2137–46. [PubMed: 19528474]

81. Nagler C, Zanker KS, Dittmar T. Cell fusion, drug resistance and recurrence CSCs. Adv Exp Med Biol. 2011;714:173–82. [PubMed: 21506014]

82. Dittmar T, Nagler C, Schwitalla S, Reith G, Niggemann B, Zanker KS. Recurrence cancer stem cells--made by cell fusion? Med Hypotheses. 2009;73:542–7. [PubMed: 19564079]

83. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100:3983–8. [PMCID: PMC153034] [PubMed: 12629218]

84. Al-Hajj M. Cancer stem cells and oncology therapeutics. Curr Opin Oncol. 2007;19:61–4. [PubMed: 17133114]

85. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Herceptin Adjuvant (HERA) Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353:1659–72. [PubMed: 16236737]

86. Perez EA, Palmieri FM, Brock SM. Trastuzumab. Cancer Treat Res. 2009;151:181–96. [PubMed: 19593513]

87. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene. 2008;27:6120–30. [PMCID: PMC2602947] [PubMed: 18591932]

88. Nakanishi T, Chumsri S, Khakpour N, Brodie AH, Leyland-Jones B, Hamburger AW, et al. Side-population cells in luminal-type breast cancer have tumour-initiating cell properties, and are regulated by HER2 expression and signalling. Br J Cancer. 2010;102:815–26. [PMCID: PMC2833247] [PubMed: 20145614]

89. Magnifico A, Albano L, Campaner S, Delia D, Castiglioni F, Gasparini P, et al. Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab. Clin Cancer Res. 2009;15:2010–21. [PubMed: 19276287]

90. Hill R, Wu H. PTEN, stem cells, and cancer stem cells. J Biol Chem. 2009;284:11755–9.

[PMCID: PMC2673242] [PubMed: 19117948]

91. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: Implications for targeted therapy. Cancer Res. 2008;68:5878–87. [PubMed: 18632642]

92. Nahta R, Hung MC, Esteva FJ. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res. 2004;64:2343–6. [PubMed: 15059883]

93. Baselga J, Swain SM. CLEOPATRA: A phase III evaluation of pertuzumab and trastuzumab for HER2-positive metastatic breast cancer. Clin Breast Cancer. 2010;10:489–91. [PubMed: 21147694]

94. Anonymous Trial watch: ERBB2 dimerization inhibitor meets end point in breast cancer trial. Nat Rev Drug Discov. 2011;10:648.

95. Montemurro F, Valabrega G, Aglietta M. Lapatinib: A dual inhibitor of EGFR and HER2 tyrosine kinase activity. Expert Opin Biol Ther. 2007;7:257–68. [PubMed: 17250463]

96. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28:803–14. [PubMed: 19060928]

97. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, et al. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proc Natl Acad Sci U S A. 2011;108:5021–6. [PMCID: PMC3064360] [PubMed: 21385943]

98. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol. 2002;20:719–26. [PubMed: 11821453]

99. Ocana A, Amir E. Irreversible pan-ErbB tyrosine kinase inhibitors and breast cancer: Current status and future directions. Cancer Treat Rev. 2009;35:685–91. [PubMed: 19733440]

100. Rabindran SK, Discafani CM, Rosfjord EC, Baxter M, Floyd MB, Golas J, et al. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. Cancer Res. 2004;64:3958–65. [PubMed: 15173008]

101. Valabrega G, Capellero S, Cavalloni G, Zaccarello G, Petrelli A, Migliardi G, et al. HER2-positive breast cancer cells resistant to trastuzumab and lapatinib lose reliance upon HER2 and are sensitive to the multitargeted kinase inhibitor sorafenib. Breast Cancer Res Treat. 2011;130:29–40. [PubMed: 21153051]

102. Nahta R, O'Regan RM. Evolving strategies for overcoming resistance to HER2-directed therapy: Targeting the PI3K/Akt/mTOR pathway. Clin Breast Cancer. 2010;10:S72–8. [PubMed: 21115425]

103. Chakrabarty A, Sanchez V, Kuba MG, Rinehart C, Arteaga CL. Breast cancer special feature: Feedback upregulation of HER3 (ErbB3) expression and activity attenuates antitumor effect of PI3K inhibitors. Proc Natl Acad Sci U S A. 2011 [In press]

104. Leighl NB, Dent S, Clemons M, Vandenberg TA, Tozer R, Warr DG, et al. A Phase 2 study of perifosine in advanced or metastatic breast cancer. Breast Cancer Res Treat. 2008;108:87–92. [PubMed: 17458693]

105. Morrow PK, Wulf GM, Ensor J, Booser DJ, Moore JA, Flores PR, et al. Phase I/II study of

trastuzumab in combination with everolimus (RAD001) in patients with HER2-overexpressing metastatic breast cancer who progressed on trastuzumab-based therapy. J Clin Oncol. 2011;29:3126–32. [PMCID: PMC3157979] [PubMed: 21730275]

106. Zhou P, Fernandes N, Dodge IL, Reddi AL, Rao N, Safran H, et al. ErbB2 degradation mediated by the co-chaperone protein CHIP. J Biol Chem. 2003;278:13829–37. [PubMed: 12574167]

107. Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A. 2002;99:12847–52. [PMCID: PMC130548] [PubMed: 12239347]

108. Tikhomirov O, Carpenter G. Identification of ErbB-2 kinase domain motifs required for geldanamycin-induced degradation. Cancer Res. 2003;63:39–43. [PubMed: 12517775]

109. Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. Proc Natl Acad Sci U S A. 2000;97:10832–7. [PMCID: PMC27109] [PubMed: 10995457]

110. Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem. 2002;277:39858–66. [PubMed: 12176997]

111. Prince T, Sun L, Matts RL. Cdk2: A genuine protein kinase client of Hsp90 and Cdc37. Biochemistry. 2005;44:15287–95. [PubMed: 16285732]

112. Bedin M, Catelli MG, Cabanie L, Gaben AM, Mester J. Indirect participation of Hsp90 in the regulation of the cyclin E turnover. Biochem Pharmacol. 2009;77:151–8. [PubMed: 18977205]

113. Lerdrup M, Bruun S, Grandal MV, Roepstorff K, Kristensen MM, Hommelgaard AM, et al. Endocytic down-regulation of ErbB2 is stimulated by cleavage of its C-terminus. Mol Biol Cell. 2007;18:3656–66. [PMCID: PMC1951740] [PubMed: 17626164]

114. Lerdrup M, Hommelgaard AM, Grandal M, van Deurs B. Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. J Cell Sci. 2006;119:85–95. [PubMed: 16352662]

115. Raja SM, Clubb RJ, Ortega-Cava C, Williams SH, Bailey TA, Duan L, et al. Anticancer activity of Celastrol in combination with ErbB2-targeted therapeutics for treatment of ErbB2-overexpressing breast cancers. Cancer Biol Ther. 2011;11:263–76. [PMCID: PMC3047084] [PubMed: 21088503]

116. Hwang M, Moretti L, Lu B. HSP90 inhibitors: Multi-targeted antitumor effects and novel combinatorial therapeutic approaches in cancer therapy. Curr Med Chem. 2009;16:3081–92. [PubMed: 19689285]

117. Chandarlapaty S, Scaltriti M, Angelini P, Ye Q, Guzman M, Hudis CA, et al. Inhibitors of HSP90 block p95-HER2 signaling in Trastuzumab-resistant tumors and suppress their growth. Oncogene. 2010;29:325–34. [PMCID: PMC3057066] [PubMed: 19855434]

118. Scaltriti M, Serra V, Normant E, Guzman M, Rodriguez O, Lim AR, et al. Antitumor activity of the Hsp90 inhibitor IPI-504 in HER2-positive trastuzumab-resistant breast cancer. Mol Cancer Ther. 2011;10:817–24. [PubMed: 21383049]

119. Modi S, Stopeck A, Linden H, Solit D, Chandarlapaty S, Rosen N, et al. HSP90 Inhibition is effective in breast cancer: A phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab. Clin Cancer Res. 2011;17:5132–9. [PubMed: 21558407]

120. Modi S, Stopeck AT, Gordon MS, Mendelson D, Solit DB, Bagatell R, et al. Combination of

trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: A phase I dose-escalation study. J Clin Oncol. 2007;25:5410–7. [PubMed: 18048823]

121. Arteaga CL. Why is this effective HSP90 inhibitor not being developed in HER2+ breast cancer? Clin Cancer Res. 2011;17:4919–21. [PMCID: PMC3221729] [PubMed: 21670086]

122. Barok M, Tanner M, Koninki K, Isola J. Trastuzumab-DM1 causes tumour growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells *in vivo*. Breast Cancer Res. 2011;13:R46. [PMCID: PMC3219209] [PubMed: 21510863]

123. Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res. 2008;68:9280–90. [PubMed: 19010901]

124. Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W, et al. Phase I study of trastuzumab-DM1, a HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. J Clin Oncol. 2010;28:2698–704. [PubMed: 20421541]

125. Burris HA, 3rd, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, Limentani S, et al. Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. J Clin Oncol. 2011;29:398–405. [PubMed: 21172893]

126. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: Novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol. 2001;21:3995–4004. [PMCID: PMC87062] [PubMed: 11359907]

127. Yardley DA, Raefsky E, Castillo R, Lahiry A, Locicero R, Thompson D, et al. Phase II study of neoadjuvant weekly nab-paclitaxel and carboplatin, with bevacizumab and trastuzumab, as treatment for women with locally advanced HER2(+) breast cancer. Clin Breast Cancer. 2011;11:297–305. [PubMed: 21729666]

128. Jager M, Schoberth A, Ruf P, Hess J, Lindhofer H. The trifunctional antibody ertumaxomab destroys tumor cells that express low levels of human epidermal growth factor receptor 2. Cancer Res. 2009;69:4270–6. [PubMed: 19435924]

129. Kiewe P, Hasmuller S, Kahlert S, Heinrigs M, Rack B, Marme A, et al. Phase I trial of the trifunctional anti-HER2 x anti-CD3 antibody ertumaxomab in metastatic breast cancer. Clin Cancer Res. 2006;12:3085–91. [PubMed: 16707606]

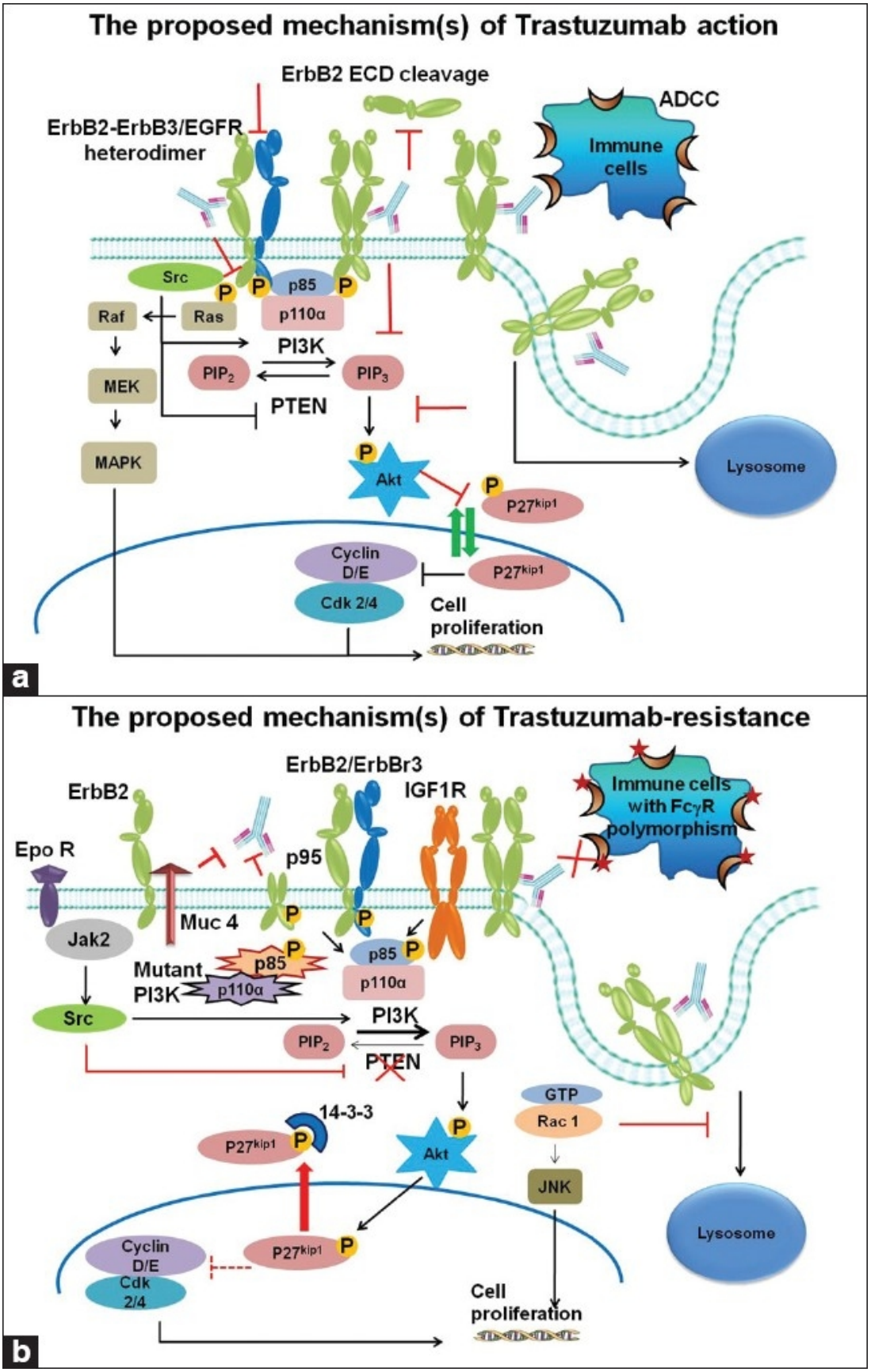
130. Tanner M, Kapanen AI, Junttila T, Raheem O, Grenman S, Elo J, et al. Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. Mol Cancer Ther. 2004;3:1585–92. [PubMed: 15634652]

131. Koninki K, Barok M, Tanner M, Staff S, Pitkanen J, Hemmila P, et al. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. Cancer Lett. 2010;294:211–9. [PubMed: 20193978]

132. Lee-Hoeflich ST, Pham TQ, Dowbenko D, Munroe X, Lee J, Li L, et al. PPM1H is a p27 phosphatase implicated in trastuzumab resistance. Cancer Discov. 2011;1:326–37.

Figures and Tables

Figure 1


The proposed mechanism(s) of Trastuzumab action
ErbB2 ECD cleavage
ErbB2-ErbB3/EGFR heterodimer
ADCC
Immune cells
Src
p85
p110α
Raf
Ras
PI3K
PIP2
PIP3
MEK
PTEN
MAPK
Akt
P27kip1
Lysosome
Cyclin D/E
Cdk 2/4
Cell proliferation
a
The proposed mechanism(s) of Trastuzumab-resistance
ErbB2/ErbBr3
ErbB2
IGF1R
Immune cells with FcγR polymorphism
Epo R
p95
Muc 4
Jak2
Mutant PI3K
p85
p110α
Src
PI3K
PIP2
PIP3
PTEN
14-3-3
P27kip1
Akt
GTP
Rac 1
JNK
Lysosome
Cyclin D/E
Cdk 2/4
Cell proliferation
b

(a) Summary of the proposed mechanism(s) of Trastuzumab action. The therapeutic effects of Trastuzumab are thought to be mediated through its ability to activate ADCC (*in vivo*) as well as cytostasis, induced via p27kip1-mediated cell cycle arrest, which can result from its effects on the following upstream pathways – (i) ErbB2 endocytosis, ubiquitinylation, and degradation; (ii) blocking dimerization-dependent activation of ErbB2 with EGFR or ErbB3; (iii) inhibition of PI3K-Akt signaling; (iv) blocking ErbB2-Src interaction to reverse Src-mediated phosphorylation (inactivating) of PTEN to reactivate PTEN; (v) inhibition of metalloprotease-induced shedding of the ErbB2-extracellular domain, leading to the prevention of constitutive signaling through p95ErbB2, and (vi) immune effector–mediated cytolysis (b) The proposed mechanism(s) that causes Trastuzumab-resistance. The potential factors that can cause resistance to Trastuzumab are: (i) PI3K hyperactivation, which can occur via multiple pathways, such as, signaling from alternative growth factor receptors (EGFR, ErbB3, and IGF-1R or p95ErbB2), PTEN-loss (through genetic or epigenetic mechanisms) or protein inactivation (through mechanisms such as oxidation, Src-dependent phosphorylation, and ubiquitin-dependent degradation), and PI3K mutations; (ii) Cyclin E dysregulation, which can occur due to overexpression of Cyclin E or downregulation of nuclear p27kip1; (iii) reduced endocytosis and lysosomal routing of ErbB2 receptors, which can be caused due to hyperactivation of c-Src non-receptor tyrosine kinase or hyperactivation of Rac1 GTPase; (iv) loss of binding of Fcγ receptors on immune effector cells to the Fc portion of Trastuzumab due to Fcγ receptor polymorphisms.

Table 1

Target	Agent	Resistance mechanism targeted	Phase of clinical development	Combination with Trastuzumab	Pharmaceutical source
ErbB2/ErbB3	Pertuzumab	Alternative GFR signaling	II	Yes	Genentech (San Franciso,CA)
EGFR/ErbB2	Cetuximab	Alternative GFR signaling	II	Yes	Bristol-Myers Squibb (New York, NY)
EGFR/ErbB2	Lapatinib	Alternative GFR signaling	II	Yes	GlaxoSmithKline (Research Triangle Park, NC
IGF1R	NVP-AEW541	Alternative GFR signaling	Preclinical		Novartis (Basel, Switzerland)
PI3K inhibitor	XL147	Downstream signaling	I-II	Yes, Paclitaxel	Exelisis (San Franciso,CA)
Akt	Perifosine	Downstream signaling	II		Keryx Biophamaceuticals (New York, NY)
Akt	MK2206	Downstream signaling	I	Yes, Lapatinib	Merck (Whitehouse station,NJ)
mTOR	RAD001	Downstream signaling	II	Yes	Novartis (New York, NY)
mTOR	AP23573	Downstream signaling	I	Yes	Ariad (Cambridge, MA)
Aromatase inhibitor	Aromasin	Downstream signaling	III	Yes	GlaxoSmithKline (Research Triangle Park, NC
HSP90 inhibitor	NYP-AUY922	All	I-II	Yes	Novartis (New York, NY)
HSP90 inhibitor	Alvespimycin	All	I	Yes, Paclitaxel	Bristol-Myers Squibb (New York, NY)
HSP90 inhibitor	Tanespimycin	All	II	Yes	Bristol-Myers Squibb (New York, NY)
ErbB2	Trastuzumab-DM1	All	II		Genentech (San Francisoo,CA)
Bevacizumab	Angiogenesis inhibitor	Angiogenesis	II	Yes, Paclitaxel and Carboplatin	Genentech (San Francisoo,CA)
ErbB2 and Fc RI	MDX-H210	Immune	II		Medarex Inc.(Annandale,NJ)
ErbB2 and Fc RIII	2B1	Immune	II		Chiron Corporation (Emeryville,CA)
ErbB2 recombinant therapeutic vaccine	Recombinant vaccine	Immune	I-II		Corixa Corporation (Seattle,WA) and GlaxoSmithKline (Research Triangle Park, NC)
DNA vaccine	AutoVac	Immune	II		Pharmexa (Horsholm Denmark)
Immunosuppression	Rapamune	Immune	II	Yes	Genentech (San Francisoo,CA)
Transcriptional inhibitor	E1A	All	I-II		Targeted Genetics Corporation (Seattle,WA)
Telomerase inhibitor	GRN163L	All	I	Yes	Geron Corporation (Menlo Park,CA)

New therapeutic approaches under evaluation to overcome Trastuzumab refractoriness in ErbB2-driven breast cancers

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 35, pp. 29442–29456, August 24, 2012

Mammalian Alteration/Deficiency in Activation 3 (*Ada3*) Is Essential for Embryonic Development and Cell Cycle Progression*[S]

Received for publication, May 4, 2012, and in revised form, June 22, 2012 Published, JBC Papers in Press, June 26, 2012, DOI 10.1074/jbc.M112.378901

Shakur Mohibi[‡1], Channabasavaiah Basavaraju Gurumurthy[‡§1], Alo Nag[§2], Jun Wang[‡], Sameer Mirza[‡3], Yousaf Mian[§4], Meghan Quinn[‡], Bryan Katafiasz[‡], James Eudy[‡], Sanjit Pandey[‡], Chittibabu Guda[‡], Mayumi Naramura[§¶], Hamid Band[‡§‖¶], and Vimla Band[‡¶5]

From the [‡]Department of Genetics, Cell Biology, and Anatomy and [‖]Departments of Biochemistry and Molecular Biology, Pathology and Microbiology, and Pharmacology and Neuroscience, College of Medicine, and the [¶]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-5805 and the [§]Department of Medicine, Evanston Northwestern Healthcare Research Institute, Northwestern University, Evanston, Illinois 60201

Background: Ada3 is a core component of HAT containing coactivator complexes.
Results: Germline deletion of *Ada3* is embryonic lethal, and cell deletion leads to abnormal cell cycle progression.
Conclusion: Ada3 is a critical protein at organismic and cellular level.
Significance: This study describes a novel role of Ada3, a component of HAT complexes, as a critical regulator of cell survival.

Ada3 protein is an essential component of histone acetyl transferase containing coactivator complexes conserved from yeast to human. We show here that germline deletion of *Ada3* in mouse is embryonic lethal, and adenovirus-Cre mediated conditional deletion of *Ada3* in *Ada3*$^{FL/FL}$ mouse embryonic fibroblasts leads to a severe proliferation defect which was rescued by ectopic expression of human *Ada3*. A delay in G_1 to S phase of cell cycle was also seen that was due to accumulation of Cdk inhibitor p27 which was an indirect effect of c-*myc* gene transcription control by Ada3. We further showed that this defect could be partially reverted by knocking down p27. Additionally, drastic changes in global histone acetylation and changes in global gene expression were observed in microarray analyses upon loss of Ada3. Lastly, formation of abnormal nuclei, mitotic defects and delay in G_2/M to G_1 transition was seen in *Ada3* deleted cells. Taken together, we provide evidence for a critical role of Ada3 in embryogenesis and cell cycle progression as an essential component of HAT complex.

The eukaryotic cell cycle progression depends on proper coordination of DNA replication and duplication of chromosomes to daughter cells (1), a process precisely regulated by modification of chromatin that allows the accessibility to factors involved in transcription (2). Thus, proteins involved in modulating the structure of chromatin play an important role in cell cycle progression. The post-translational modification of core histones (H2A, H2B, H3, and H4) is an essential process for altering chromatin structure (3, 4). Histone acetyl transferases (HATs)[6] and histone deacetylases are required to maintain steady state levels of acetylation (5). Several HAT enzymes, such as general control nonderepressible 5 (Gcn5), p300/CBP-associated factor (PCAF), p300, and CREB-binding protein (CBP), have been identified over the years (6, 7). Most of the HATs are part of large complexes such as the human TBP-free TAF complex (TFTC); the Spt3/Taf9/Gcn5 acetyltransferase complex (STAGA) (human homolog of yeast SAGA complex) and the Ada2a-containing (ATAC) complex that play a role in several important processes, such as cell cycle (8, 9). Additionally, previous studies from our laboratory and that of others have demonstrated the presence of p300 HAT in Ada3-containing protein complexes (10, 11). Given the combined presence of Ada3 with Gcn5 in a number of distinct HAT complexes, recent evidence for a role of Gcn5 in regulating DNA replication as well as mitosis (12–14) suggest that Ada3 may also play a role in cell cycle. Despite the range of established and potential cellular functions of Ada3 as part of multiple HAT complexes, the *in vivo* physiological role of mammalian Ada3 is not known.

We previously identified human Ada3 as a novel human papillomavirus 16 E6-binding protein (15). Human Ada3 is the

* This work was supported, in whole or in part, by National Institutes of Health Grant CA96844 and CA144027 (to V. B.) and CA87986, CA99163, CA105489, CA116552 and NCI 5U01CA151806-02 (to H. B.). This work was also supported by Department of Defense Grants W81XWH-07-1-0351 and W81XWH-11-1-0171 (to V. B.).
[S] This article contains supplemental Materials and Methods, Figs. S1–S6, and Tables S1–S3.
[1] Both authors contributed equally to this work.
[2] Present address: Dept. of Biochemistry, University of Delhi South Campus, New Delhi-110021, India.
[3] Supported by Susan G. Komen Postdoctoral Fellowship KG111248.
[4] Present address: Molecular Biology Graduate Program, Loyola University Chicago, Maywood, IL 60153.
[5] To whom correspondence should be addressed: Dept. of Genetics, Cell Biology and Anatomy, College of Medicine, 985805 Nebraska Medical Center, University of Nebraska Medical Center, Omaha, NE 68198-5805. Tel.: 402-559-8565; Fax: 402-559-7328; E-mail: vband@unmc.edu.

[6] The abbreviations used are: HAT, histone acetyltransferase; Ada3, alteration/deficiency in activation 3; hAda3, human Ada3; MEF, mouse embryonic fibroblast; Cdk, cyclin-dependent kinase; Gcn5, general control nonderepressible 5; PCAF, p300/CBP-associated factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; STAGA, Spt3/Taf9/Gcn5 acetyltransferase complex; ATAC, Ada2a-containing complex; adeno-Cre, adenovirus expressing the Cre recombinase; Rb, retinoblastoma protein; E, embryonic days; PI, propidium iodide; TBP, TATA-binding protein; TAF, TBP-associated factor.

homologue of the yeast Ada3, an essential component of the Ada transcriptional coactivator complex composed of Ada2, Ada3, and a HAT component Gcn5 (16). Genetic studies in yeast have demonstrated that Ada3 functions as a critical component of coactivator complexes that link transcriptional activators, bound to specific promoters, to histone acetylation and basal transcriptional machinery (17–19). We showed that Ada3 binds and stabilizes the tumor suppressor p53 protein and is required for p53 acetylation by p300 (20). Work from our laboratory has also shown that Ada3 is required for HAT recruitment to estrogen receptors and their transcription activation function (11). We and others have shown that Ada3 also associates with and regulates transcriptional activity of other nuclear hormone receptors, including retinoic acid receptor (21) and androgen receptor (22).

Here, we used conditional deletion of mouse *Ada3* gene to explore the physiological importance of mammalian Ada3. We demonstrate that homozygous deletion of *Ada3* is early embryonic lethal. *Ada3* deletion in $Ada3^{Flox/Flox}$ ($Ada3^{FL/FL}$) MEFs showed that Ada3 is required for efficient cell cycle progression through G_1 to S transition as well as for proper mitosis. Detailed analyses in this system revealed an Ada3-c-Myc-Skp2-p27 axis that controls G_1 to S phase progression and partly contributes to cell cycle delay upon *Ada3* deletion. Additionally, loss of Ada3 showed dramatic decrease in acetylation of core histones that are known to play an important role in cell cycle. Loss of Ada3 also resulted in several changes in gene expression as observed by microarray analyses. Notably, many of the genes affected were involved in mitosis. Taken together, we present evidence for an essential role of mammalian Ada3 in embryonic development and cell cycle progression.

EXPERIMENTAL PROCEDURES

Generation of Ada3 Gene-targeted Mice, Isolation of Mouse Embryos and PCR Genotyping—Details concerning generation of conditional *Ada3* knock-out construct and *Ada3* knock-out mouse as well as PCR genotyping strategies are described in the supplemental data.

Cell Culture Procedures and Viral Infections—Embryonic day 13.5 embryos were dissected from $Ada3^{FL/+}$ intercrossed females, and MEFs were isolated and immortalized following the 3T3 protocol (23). MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Adenoviruses expressing EGFP-Cre or enhanced green fluorescent protein (EGFP) alone were purchased from the University of Iowa (Gene Transfer Vector Core). An adenovirus dose of 50–100 MOI diluted in 4 ml of serum-free medium was added to cells in 100-mm culture dishes (at about 30% confluence) and incubated for 1 h each at room temperature and at 37 °C followed by the addition of 7 ml of complete medium. After overnight incubation at 37 °C, medium was replaced with complete medium, and cells were carried further for various experiments. To generate retroviral FLAG-h*Ada3* vector, full-length FLAG-h*Ada3* (15) was cloned into pMSCVpuro vector (Clontech). Retroviruses were generated by transiently transfecting this retroviral construct into the Phoenix ecotropic packaging cell line using the calcium phosphate co-precipitation method. The retroviruses were transduced into $Ada3^{FL/FL}$ MEFs by three infections at 12-h intervals using supernatant from transfected Phoenix cells to generate $Ada3^{FL/FL}$ MEFs expressing FLAG-hAda3. Scrambled shRNA (5′-GGTTAAA-ACCTTACGATGT-3′) or p27 shRNA (5′-GTGGAATTTCG-ACTTTCAG-3′) was introduced into $Ada3^{FL/FL}$ MEFs by using three infections at 12-h intervals of the shRNA bearing pSUPER.retro.puro (Oligoengine) retrovirus containing supernatants from Phoenix cells. Retroviral infections were carried out in the presence of 8 μg/ml Polybrene (Sigma) and were followed by selection in 2 μg/ml puromycin for 48 h until complete loss of uninfected cells.

Proliferation Assay, Colony Formation Efficiency Assay, and Cell Cycle Analysis—To perform proliferation assays, 1 day after adenovirus infection, cells were plated at different numbers in 6-well plates in triplicates (5×10^4 (for counting on day 3), 2.5×10^4 (for counting on day 5), 1.25×10^4 (for counting on day 7), and 0.625×10^4 (for counting on day 9) and counted at the indicated time points. For colony formation assay, cells 3 days after adenovirus-infection were trypsinized and plated at 1000 cells per 100-mm culture dishes in triplicates and carried for 15 more days with medium change as required. At the end of incubation, colonies in dishes were fixed and stained with crystal violet solution (0.25% crystal violet in 25% methanol) and photographed. For cell cycle analysis, 2 days after plating and adenoviral infection of 2×10^5 cells in 100-mm culture dishes, cells were synchronized by replacing the complete medium with DMEM + 0.1% FCS and incubating for 72 h. Synchronized cells were stimulated with complete medium (DMEM + 10% FCS) for various time points and harvested and stained with propidium iodide (PI) for FACS analysis. For synchronization of cells at G_2/M phase, 48 h after adenovirus infection, cells were switched to complete medium containing 125 ng/ml nocodazole for 18 h. Following synchronization, cells were washed three times with PBS and stimulated with complete medium for various time points and analyzed by FACS after PI staining.

Generation of Ada3 Monoclonal Antibody and Immunoblotting—Antibodies used in this study can be found in the supplemental data.

In Vitro Kinase Assay—In vitro kinase assay was performed using purified histone H1 (Roche Applied Science) or Rb (769) (Santa Cruz Biotechnology sc-4112) as a substrate. Adenovirus-infected MEFs were starved for 3 days and stimulated with serum. Cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM Na_4VO_3, 1 mM NaF, and protease inhibitor mixture), and cyclin-dependent kinase (Cdk) complex was recovered by immunoprecipitation with 2 μg of either anti-Cdk4 (sc-56277)/Cdk6 (sc-53638) antibodies mixture or anti-Cdk2 (sc-6248) antibody (Santa Cruz Biotechnology). Cdk4/6 or Cdk2 complexes were captured with protein G-agarose for 1 h and washed with lysis buffer followed by one wash with kinase buffer (50 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM Na_4VO_3, and 1 mM NaF). Cdk2 complex was incubated with histone H1 (2 μg) or Rb (500 ng), whereas Cdk4/6 complex was incubated with only Rb (500 ng) in kinase buffer containing 10 mM β-glycerophosphate, 33 μM ATP, and 10 μCi of [γ-^{32}P]ATP (10 mCi/ml, 6000 Ci/mmol) at room temperature for 20 min. The products were

subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF), and autoradiographed.

Analysis of the p27 Protein Turnover—*Ada3*$^{FL/FL}$ MEFs were plated in 100-mm dishes and infected with control or Cre adenoviruses. For analyzing p27 protein half-life in exponentially growing cells, 2 days after adenovirus infection, cells were treated with 50 μg/ml cycloheximide (Sigma) and harvested at the indicated time points. For analyzing p27 protein half-life in serum-starved cells, 2 days after adenovirus infection, cells were starved for 72 h in 0.1% serum-containing medium. Subsequently, 50 μg/ml cycloheximide was added to the medium, and cells were harvested at the indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. Densitometry analysis was carried out on scanned images using ImageJ software.

RNA Extraction and Quantitative Real-time PCR—TRIzol reagent (Invitrogen) was used to isolate total RNA from MEFs infected with control virus or Cre adenovirus. 2 μg of total RNA was used for reverse transcriptase reaction using SuperScript™ II reverse transcriptase (Invitrogen). Real-time PCR quantification was performed in triplicates using SYBR Green PCR master mix (Applied Biosystems) and the primers listed in supplemental Table S3. Expression levels were normalized against β-actin mRNA levels, and the results were calculated by the $\Delta\Delta C_t$ method.

Chromatin Immunoprecipitation Experiments—Approximately 0.7 million *Ada3*$^{FL/FL}$ MEFs were plated in 100-mm dishes and infected with control or Cre adenoviruses. Forty-eight hours after infection, cells were synchronized with DMEM + 0.1% FCS for 72 h and then stimulated with complete medium (DMEM + 10% FCS) for 0–60 min as indicated for each experiment in Fig. 8*C*. ChIP experiment was performed by using the ChIP-IT Express kit from Active Motif. PCR amplification was performed using primers for the c-*myc* enhancer (forward, 5′-CTAGAACCAATGCACAGAGC-3′; reverse, 5′-CTCCCAGGACAAACCCAAGC-3′) and for the *Skp2* promoter (forward, 5′-GCCATCGAGACCCCGGAGAT-3′; reverse, 5′-TGAGTCCCTTCCAGACGCTGT-3′). Control PCR was performed using primers for the c-*myc* distal site (forward, 5′-ACACACCTTGAATCCCGT-3′; reverse, 5′-CCCAGCTAGAATGAAGAAG-3′) and the *Skp2* distal site (forward, 5′-GTGCTAGCTGCTTACCTTTGT-3′; reverse, 5′-GATAAGGATGCACTCTGGGGC-3′). PCR products were analyzed on 2% agarose/Tris-acetate-EDTA gels with ethidium bromide stain. PCR of the input DNA prior to immunoprecipitation was used as a control.

Generation of Recombinant Baculoviruses and Ada3-His Expression Using Bac-to-Bac® Expression System—Ada3 baculoviral construct information and recombinant protein purification are detailed in the supplemental data.

HAT Assay—Protocol used for *in vitro* HAT assay can be found in the supplemental data.

Microarray Analyses—Protocol for microarray analyses is described in the supplemental data. The microarray data from this publication have been submitted to the GEO database and have been assigned the following Series record: GSE37542.

TABLE 1
Genotype analysis of embryos from heterozygous intercrosses

Stage	Total no. of embryos	No. (%) of embryos			
		WT	Heterozygous	KO	Resorbed
Live born	224	75 (33)	149 (66)	0	0
E12.5	14	3 (21)	5 (36)	0	6 (43)
E 9.5	15	8 (53)	2 (13)	0	5 (33)
E 8.5	44	12 (27)	27 (61)	0	5 (11)
E 3.5	15	4 (27)	7 (47)	4 (27)	0

RESULTS

Deletion of Ada3 Leads to Early Embryonic Lethality in Mice—The targeting construct generated using the recombineering technique (supplemental Fig. S1*A*; see supplemental Materials and Methods) was electroporated into an ES cell line derived from the 129/Ola strain of mice. Screening of resultant neomycin-resistant colonies yielded three correctly targeted clones (supplemental Fig. S1*B*). One positive clone was microinjected into blastocysts. The resulting chimeras transmitted the targeted allele to their progeny as verified by PCR. The neomycin cassette flanked by Frt recombination sites was removed by crossing the *Ada3*-targeted mice to FlpE recombinase transgenic mice (B6.Cg-Tg (ACTFLPe) 9205Dym/J; stock number 005703). Homozygous *Ada3*$^{FL/FL}$ mice were viable and fertile and exhibited no gross abnormalities when compared with *Ada3*$^{FL/+}$ or *Ada3*$^{+/+}$ controls. To achieve *Ada3* deletion, heterozygous *Ada3*-targeted mice (*Ada3*$^{FL/+}$ mice) were bred with transgenic mice expressing the adenovirus EIIa promoter-driven Cre (B6.FVB-Tg (EIIa-Cre) C5379Lmgd/J). EIIa directs Cre expression in a wide range of tissues including germ cells. Heterozygous *Ada3*-targeted, Cre transgene-positive mice were crossed to C57BL/6J (wild-type) mice to generate heterozygous *Ada3*-deleted, Cre transgene-negative (*Ada3*$^{+/-}$) mice. Heterozygous *Ada3*$^{+/-}$ mice of a mixed 129/Sv × C57BL/6 background were viable and fertile, and their median life span of more than 18 months was comparable with that of their control littermates (data not shown). Heterozygous *Ada3*$^{+/-}$ mice were intercrossed to obtain homozygous *Ada3*-null mice. No *Ada3*$^{-/-}$ mice were observed among 224 live born pups screened (Table 1). The ratio of wild type to heterozygous offspring was 1:2, indicating that the loss of one *Ada3* allele does not lead to haploinsufficiency in mice.

To assess the specific period of developmental failure in the *Ada3* knock-out mice, embryos derived from *Ada3*$^{+/-}$ intercrosses were genotyped at different stages of gestation using a duplex PCR method (supplemental Fig. S1, *C* and *D*). Because no homozygous mutant embryos were recovered beyond embryonic day 8.5 (E8.5; Table 1), blastocysts were isolated at 3.5 days postcoitum and genotyped directly by PCR (supplemental Fig. S1*E*). When compared with blastocysts of *Ada3*$^{+/+}$ and *Ada3*$^{+/-}$ genotypes, *Ada3*$^{-/-}$ blastocysts that attached to culture dishes showed severe growth retardation of the trophoblast layer, and the inner cell mass was absent (supplemental Fig. S1*F*). PCR analysis revealed that ~25% of blastocysts analyzed were null for *Ada3* (Table 1). These results demonstrate that Ada3 plays a critical role in early embryogenesis in mice. The failure of *Ada3*$^{-/-}$ embryos to remain viable beyond E3.5 suggests a potential role of Ada3 in cell proliferation because

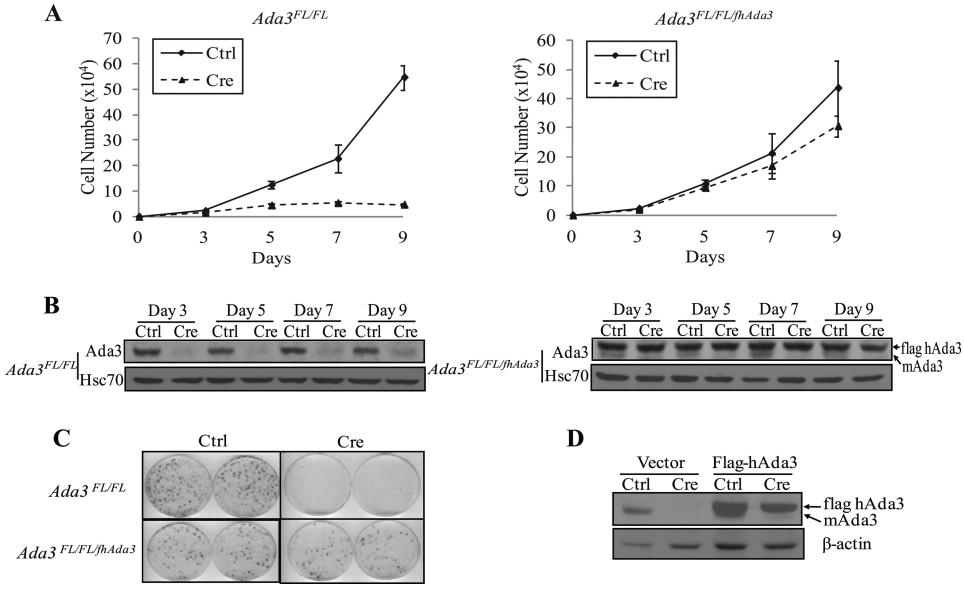


FIGURE 1. **Ablation of Ada3 causes proliferation defect in MEFs.** *A*, growth curves of *Ada3*$^{FL/FL}$ (*left*) and *Ada3*$^{FL/FL/fhAda3}$ (*right*) MEFs after control adenovirus (*Ctrl*)or Cre adenovirus (*Cre*) infection. Data are mean ± S.E. from three independent experiments performed in triplicates. *B*, Ada3 protein levels at different time points after Cre adenovirus infection. Note that reconstituted control cells express both mouse (*mAda3*; *lower band*) and human (FLAG hAda3; *upper band*) proteins, whereas only hAda3 is seen in Cre adenovirus-infected cells. *C*, colony formation assay. Crystal violet staining of the indicated cells infected with control virus or Cre adenovirus grown for 10 days is shown. *D*, Western blotting of lysates from *C* showing exogenous and endogenous Ada3.

extensive cellular proliferation occurs during this early stage of embryogenesis (see later sections).

Ada3 Is Ubiquitously Expressed in Adult Mouse Tissues—Embryonic lethality of *Ada3*$^{-/-}$ mice suggested a potential role of Ada3 in growth and development of many tissues. To examine whether Ada3 is expressed in adult tissues, we analyzed the relative levels of Ada3 protein expression in a range of adult mouse tissues. For this purpose, lysates from various tissues of 8-week-old wild-type mice were subjected to immunoblotting using an anti-Ada3 monoclonal antibody generated in our laboratory (see supplemental Materials and Methods). As seen in supplemental Fig. S2, Ada3 is ubiquitously expressed in all the tissues with higher levels seen in the mammary gland, lung, and thymus. These results suggest potentially ubiquitous functional roles of Ada3 and are consistent with embryonic lethal phenotype of its germline deletion.

Conditional Ada3 Deletion in MEFs Leads to Proliferation Arrest—Given the embryonic lethality as a result of *Ada3* deletion, we resorted to a cellular model of conditional *Ada3* deletion to investigate its roles at the cellular level. For this purpose, we generated *Ada3*$^{FL/FL}$ mice by interbreeding *Ada3*$^{FL/+}$ mice and established MEFs from these mice. Conditional *Ada3* deletion was obtained by infecting *Ada3*$^{FL/FL}$ MEFs with an adenovirus expressing the Cre recombinase (adeno-Cre), with adeno-GFP serving as a control. To assess the effects of Ada3 on cell proliferation, equal numbers of control- and adeno-Cre-infected MEFs were plated a day after adenoviral infection, and cells were counted at the indicated time points up to 9 days. Notably, *Ada3*-deleted MEFs exhibited a significantly slower rate of proliferation when compared with control MEFs (Fig. 1*A*, *left*). To confirm that the defect in cell proliferation was specifically due to depletion of Ada3, we generated *Ada3*$^{FL/FL/hAda3}$ MEFs by retrovirally introducing human *Ada3* (h*Ada3*) with an N-terminal FLAG tag into *Ada3*$^{FL/FL}$ MEFs. These transfectants were verified to be expressing the exogenous FLAG-tagged Ada3 protein (Fig. 1*B*). Similar to *Ada3*$^{FL/FL}$ MEFs, adeno-Cre infection of these cells led to deletion of endogenous *Ada3* and loss of its protein product (Fig. 1*B*). Notably, however, Cre-mediated deletion of *Ada3* in *Ada3*$^{FL/FL/hAda3}$ MEFs had a minimal effect on the proliferation of MEFs, whereas similar treatment of *Ada3*$^{FL/FL}$ MEFs led to reduction in the rate of proliferation; thus, the proliferative defect induced by deletion of mouse *Ada3* in MEFs was rescued by exogenous hAda3 (Fig. 1*A*, *right*). Colony formation efficiency assay, as an independent method to measure the extent of cell proliferation, further confirmed the proliferative defect of *Ada3*-deleted MEFs that could be rescued by reconstitution with exogenous hAda3 (Fig. 1, *C* and *D*).

Ada3 Is Required for Cell Cycle Progression through G_1 to S Phase—We reasoned that the proliferation defect upon *Ada3* deletion in MEFs could reflect a role of Ada3 in cell cycle progression. To directly examine whether Ada3 plays a role in cell cycle progression, *Ada3*$^{FL/FL}$ MEFs were infected with control and Cre adenoviruses, arrested in G_0/G_1 by serum deprivation for 72 h, and then synchronously released into cell cycle by serum stimulation. FACS-based cell cycle analysis of propidium iodide-stained cells showed significant delay in G_1 to S progression in *Ada3*-deleted MEFs when compared with control MEFs (Fig. 2*A*). Of note, the relative distribution of S phase in *Ada3*-null MEFs after 20 h of serum stimulation was about half (31.6 ± 2.33 S.E. %) of the control virus-infected MEFs (56.05 ± 4.71 S.E. %) (Fig. 2*B*). These results demonstrate that conditional deletion of *Ada3* leads to delay in G_1 to S progression in MEFs, indicating an essential role of Ada3 in efficient G_1/S progression.

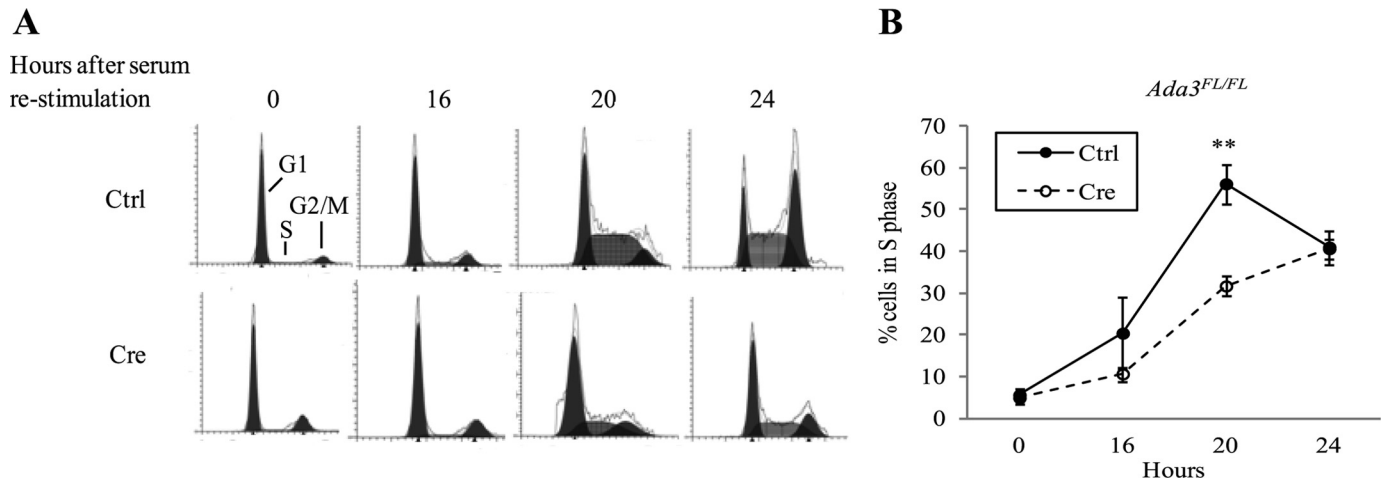


FIGURE 2. ***Ada3* disruption delays G_1 to S transition in MEFs.** *A*, control (*Ctrl*)- or Cre- infected *Ada3*$^{FL/FL}$ MEFs were serum-starved for 72 h and then released from synchrony as described under "Experimental Procedures" and processed for PI staining followed by FACS analysis. Cells in different phases of the cell cycle are shown from a representative experiment. *B*, graph derived from three independent experiments performed as in *A*, showing the proportion of cells entering into S phase at the indicated times after serum restimulation. *Error bars* are mean ± S.E. from three independent experiments (**, $p = 0.0096$, two-tailed Student's *t* test).

Elevated p27^{Kip1} *Levels and Impaired Rb Phosphorylation upon Conditional Ada3 Deletion*—Given the delay in G_1/S progression imposed by induced Ada3 deficiency, we examined the status of key proteins known to control the G_1/S transition. A well established and critical event during G_1 to S progression is the phosphorylation of Rb by Cdk complexes (particularly complexes containing Cyclins D, E, or A), such as Cdk4/6 and Cdk2 (24, 25); phosphorylation of Rb leads to its release from Rb/E2F complexes, relieves E2Fs from repression, and facilitates the expression of E2F-responsive genes important for S phase progression (24, 25). Furthermore, degradation of Cdk inhibitors, such as p27, is required for progression of cells from G_1 to S phase (26, 27). Therefore, we carried out Western blotting of cell lysates obtained from control *versus* conditional *Ada3*-deleted MEFs released into synchronous cell cycle progression to assess the levels of proteins relevant to the G_1 to S phase transition. Notably, although minimal to no changes were observed in the levels of Cdk2, Cdk4, Cdk6, p16, p21, cyclin E, and cyclin D, a significant increase in p27 levels, a delay in the cell cycle-associated increase in cyclin A levels, and a lower level of Rb phosphorylation were observed in MEFs upon *Ada3* deletion when compared with control cells (Fig. 3*A*).

In view of increased levels of p27 without a significant change in the levels of Cdk proteins in cells with *Ada3* deletion, we assessed the level of Cdk2 kinase activity using an i*n vitro* kinase assay on immunoprecipitates from cells. Although the Cdk4/6 kinase activity was comparable between control- and adeno-Cre-infected MEFs (Fig. 3*B*), the level of Cdk2 kinase activity was substantially reduced in Cre-infected MEFs when compared with control MEFs (Fig. 3*B*). These results suggest the potential reduction of Cdk2 kinase activity in the *Ada3*-deleted cells as a result of an increase in the levels of p27, accounting for defective Rb phosphorylation.

Accumulation of p27 upon Ada3 Deletion Is due to Increased Stability of p27—As accumulation of p27 levels upon *Ada3* deletion appeared to be functionally important, we examined whether this accumulation was at the transcriptional or post-transcriptional level. Real-time PCR analysis showed that serum stimulation resulted in a marked reduction in the levels of *Cdkn1b* mRNA in both the control-infected and the Cre-infected cells (Fig. 4*A*); furthermore, the levels of *Cdkn1b* mRNA at various time points after serum addition remained comparable between the two cell populations, reinforcing the idea that the increase in p27 protein levels in *Ada3*-deleted cells was likely to be at a post-transcriptional level. As alterations in protein stability are a prominent mechanism to control Cdk inhibitor levels (28), we compared the half-life of p27 protein in WT *versus Ada3*-deleted MEFs using two distinct experimental formats; the first one utilized exponentially growing cultures, whereas the second one utilized cells first arrested in G_1 by serum deprivation for 72 h followed by synchronous release into cell cycle by serum addition. In each case, *Ada3*$^{FL/FL}$ MEFs infected with control or Cre adenoviruses were treated with cycloheximide to block new protein synthesis, and p27 levels in cell lysates following cycloheximide treatment were quantified using immunoblotting at various time points. Previous work has shown that p27 half-life in exponentially growing MEFs is about 3 h and increases to about 8 h in serum-starved cells (29). We found the p27 half-life in cells infected with control adenovirus was consistent with published results, *i.e.* approximately 2 h and 40 min in exponentially growing MEFs, whereas in growth-arrested cells, half-life was approximately 3 h and 30 min (Fig. 4, *B–E*). Notably, in both experimental formats, we observed a substantial increase in p27 protein half-life upon Cre-dependent *Ada3* deletion, with approximate half-lives of 4 h and 10 min and 6 h in exponentially growing *versus* synchronous culture formats, respectively. These results strongly support our conclusion that accumulation of p27 protein upon *Ada3* deletion is due to its increased stability.

Depletion of p27 from Conditionally Deleted Ada3 MEFs Causes a Partial Rescue of G_1/S Progression Defects—Reduced activity of the p27 target Cdk2 in *Ada3*-deleted MEFs strongly suggested a role for p27 in defective cell cycle progression in these cells. To directly establish whether this is the case, we generated stable p27 knockdown *Ada3*$^{FL/FL}$ MEFs (*Ada3*$^{FL/FL/p27shRNA}$) by infecting *Ada3*$^{FL/FL}$ MEFs with a retro-

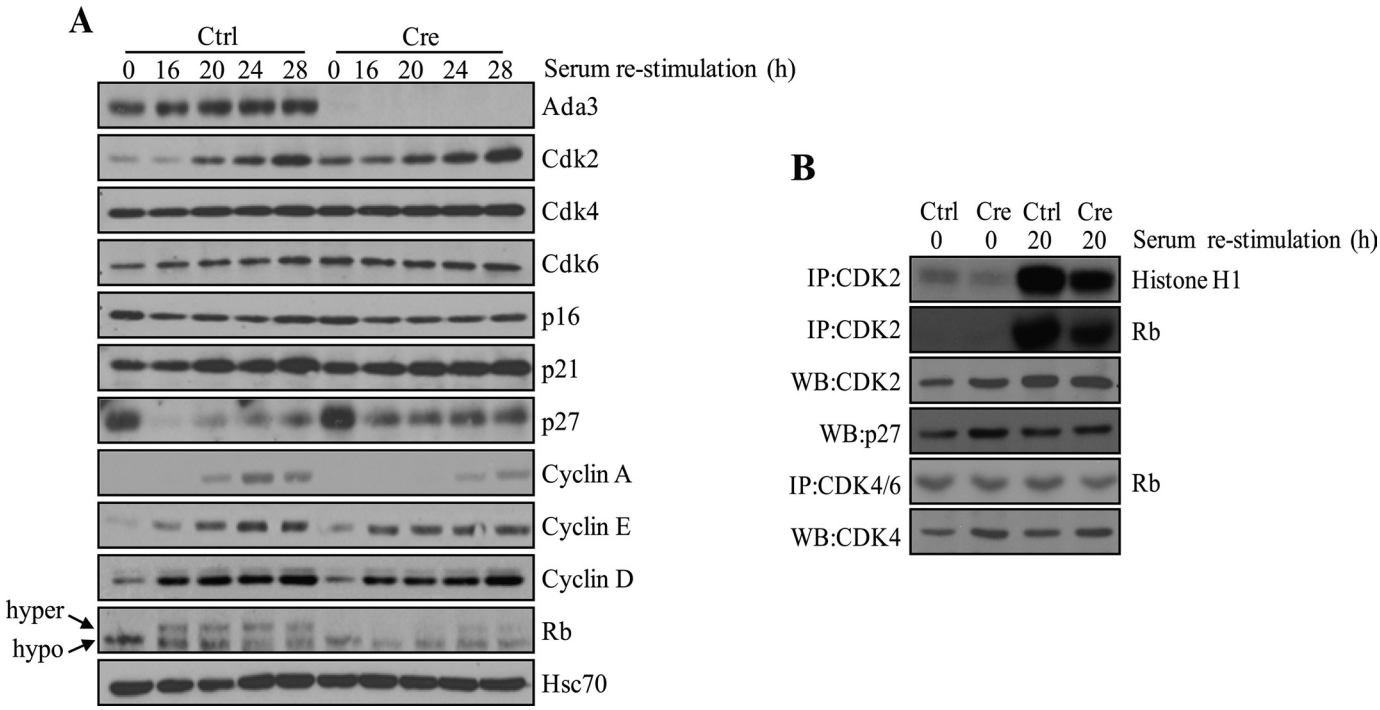


FIGURE 3. **Effect of Ada3 depletion on expression of cell cycle regulator proteins and Cdk2 kinase activity.** *A*, *Ada3*$^{FL/FL}$ MEFs infected with control (*Ctrl*) and Cre adenoviruses serum-starved for 72 h, released from synchrony as described under "Experimental Procedures," and processed for immunoblot analysis of the indicated cell cycle proteins. *hyper*, hyperphosphorylated; *hypo*, hypophosphorylated. *B*, anti-Cdk2 or anti-Cdk4/6 immunoprecipitations performed using 300-μg extracts of *Ada3*$^{FL/FL}$ MEFs infected with control or Cre adenovirus were subjected to *in vitro* kinase assay using histone H1 or Rb as a substrate. *WB*, Western blot; *IP*, immunoprecipitation.

virus expressing a p27-specific shRNA followed by selection in puromycin, which resulted in a significant knockdown of p27 expression in these cells (Fig. 5*A*). Next, we infected the *Ada3*$^{FL/FL/p27shRNA}$ MEFs with control or Cre adenovirus and analyzed these for cell cycle progression using serum deprivation followed by serum stimulation, as above (Fig. 5*B*). Notably, a partial but clear rescue of the G_1/S delay was observed in p27 shRNA-expressing cells, as seen by a much larger percentage of cells entering the S phase (41.4 ± 3.5 S.E. % in p27shRNA expressing conditionally deleted *Ada3* MEFs *versus* 31.6 ± 2.33 S.E. % in Ada3-deleted MEFs at 20 h; compare Fig. 5*C* with Fig. 2*B*). Importantly, the levels of cyclin A, which is known to be expressed during G_1/S transition and to peak in the S phase, as well as hyperphosphorylation of Rb, were essentially fully rescued by p27 shRNA knockdown (Fig. 5*D*; compare with Fig. 3*A*). Taken together, these results clearly demonstrate an important role of Ada3-dependent control of p27 levels in promoting cell cycle progression.

Deletion of Ada3 Leads to Reduced Protein and mRNA Levels of Skp2 and c-Myc—Given the causal link established above between p27 accumulation and G_1/S cell cycle delay upon *Ada3* deletion, we wished to examine the molecular mechanism by which loss of Ada3 promotes p27 stability. Published studies have established a major role of Skp2-containing E3 ubiquitin ligases in regulating p27 protein turnover during cell cycle progression (30). As *Skp2* is a transcriptional target of c-Myc (31) and Ada3-containing STAGA complex has been shown to increase *myc* mRNA transcription (32, 33), the possibility of an Ada3-c-Myc-Skp2-p27 regulatory pathway appeared to be a plausible mechanism for our findings. To explore this hypothesis, we first examined the effects of *Ada3* deletion on the levels of Skp2 mRNA (real-time PCR) and protein (immunoblotting). For this purpose, *Ada3*$^{FL/FL}$ cells infected with control or Cre adenovirus were serum-deprived and released into synchronous cell cycle progression by adding serum followed by analyses of Skp2 mRNA and protein at various time points. Notably, Skp2 mRNA and protein levels were substantially lower at each comparable time point in adeno-Cre-infected *versus* control MEFs (Fig. 6, *A* and *B*). These results indicate that *Ada3* deletion indeed leads to reduction in Skp2 levels and that this effect is likely due to reduced *Skp2* gene transcription.

Next, we asked whether *Ada3* deletion alters c-Myc mRNA levels and whether Ada3 directly binds to c-*myc* promoter. Indeed, analysis of control *versus Ada3*-deleted MEFs stimulated with serum to undergo cell cycle progression demonstrated that c-Myc mRNA as well as protein levels were significantly lower at each time point examined upon deletion of *Ada3* from cells (Fig. 6, *C* and *D*). Consistent with this, we observed lower occupancy of mouse *Skp2* promoter by c-Myc upon deletion of *Ada3*, which supports our results (supplemental Fig. S3). Finally, to establish that Ada3 indeed participates in the enhancement of *myc* gene transcription, we carried out ChIP analysis to assess whether Ada3 is recruited to c-*myc* enhancer during cell cycle progression. Indeed, a rapid recruitment of Ada3, as well as RNA polymerase II (used as positive control), to c-*myc* enhancer at −1.4 kb relative to transcription start site (but not to a distal site at −5 kb) was seen upon serum stimulation of MEFs (Fig. 6*E*). As expected, we did not detect any signals after immunoprecipitation with anti-Ada3 antibody in cells infected with adeno-Cre. These results therefore sup-

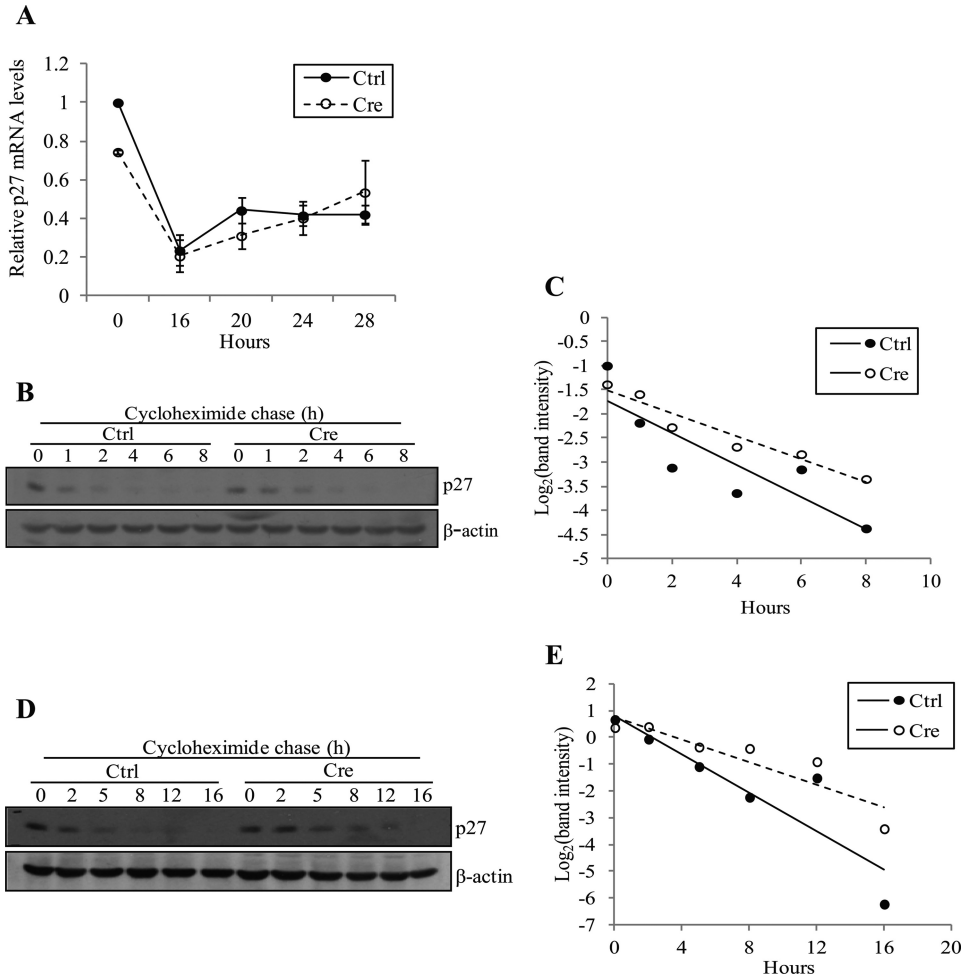


FIGURE 4. **Deletion of *Ada3* does not affect p27 transcription but extends p27 protein half-life.** *A*, unaltered p27 mRNA levels after *Ada3* deletion. Real-time RT-PCR analysis of p27 mRNA levels from cells as treated in Fig. 2 was performed. Signals were normalized to β-actin levels and plotted relative to the level of p27 mRNA in starved control (*Ctrl*) cells. *Error bars* show mean ± S.E. from three independent experiments. *B–E*, *Ada3* deletion in MEFs extends p27 half-life. *B*, 48 h after adenovirus infection, MEFs were treated with 50 μg/ml cycloheximide and harvested at the indicated time points, and p27 and β-actin protein levels were analyzed by immunoblotting. *C*, the intensity of p27 bands was quantified by densitometry, normalized to β-actin using ImageJ software, and plotted against the time of cycloheximide treatment. Each decrease of 1 unit of log 2 is equivalent to one half-life. The lines were generated by linear regression formula. *D*, after 48 h of adenovirus infection, MEFs were starved using 0.1% serum-containing medium for 72 h and subsequently treated with 50 μg/ml cycloheximide and harvested at the indicated time points. Cell lysates were analyzed by Western blotting using antibodies against p27 and β-actin. *E*, graph made from experiment in *D* by using the same procedure as in *C*.

port the existence of a novel cell cycle-associated, Ada3-regulated signaling pathway that promotes G_1/S cell cycle progression by regulating p27 stability through Myc-dependent control of *Skp2* expression.

Ada3 Deletion Leads to Decreased Histone Acetylation—As we observed a partial rescue of G_1/S transition in *Ada3*-deleted MEFs after knockdown of p27, we speculated that Ada3 deletion-induced cell cycle arrest may involve other pathways as well. Given the known literature on Ada3 as part of HAT complexes (8, 9), we examined whether Ada3 is involved in controlling global histone acetylation. Therefore, we assessed the effect of *Ada3* deletion on lysine acetylation of various core histones. We expressed Cre recombinase in $Ada3^{FL/FL}$ MEFs and harvested protein samples from asynchronous cultures after 3 days of infection. Western blotting using antibodies against important acetylated lysine residues of all four core histones (H2A-K5, H2B-K5, H3-K9, H3-K56, and H4-K8) showed a significant reduction in acetylation at all these sites in *Ada3*-deficient MEFs when compared with control MEFs (Fig. 7*A*), indicating that Ada3 is essential in maintaining global histone acetylation.

We further examined the effect of *Ada3* deletion on acetylation of core histones after synchronizing cells in G_1 phase and subsequent release. There was a dramatic down-regulation of H3-K9 acetylation and a slight decrease in acetylation of H2B-K5 in *Ada3*-deleted MEFs when compared with control-MEFs, whereas this defect was rescued in $Ada3^{FL/FL}$ MEFs reconstituted with exogenous human FLAG-Ada3 (Fig. 7*B*), suggesting that the defect in histone acetylation seen in *Ada3*-deleted MEFs was a consequence of *Ada3* deletion. Histone acetylation has been shown to be important for deposition of histones during replication-coupled nucleosome assembly as well as for chromatin maturation following DNA replication (34, 35). Thus, the partial rescue in G_1 to S transition observed upon knockdown of p27 in *Ada3*-deficient cells could be attributed to massive histone acetylation defects, which would create difficulties for cells to undergo DNA replication and thus delay transition through S phase.

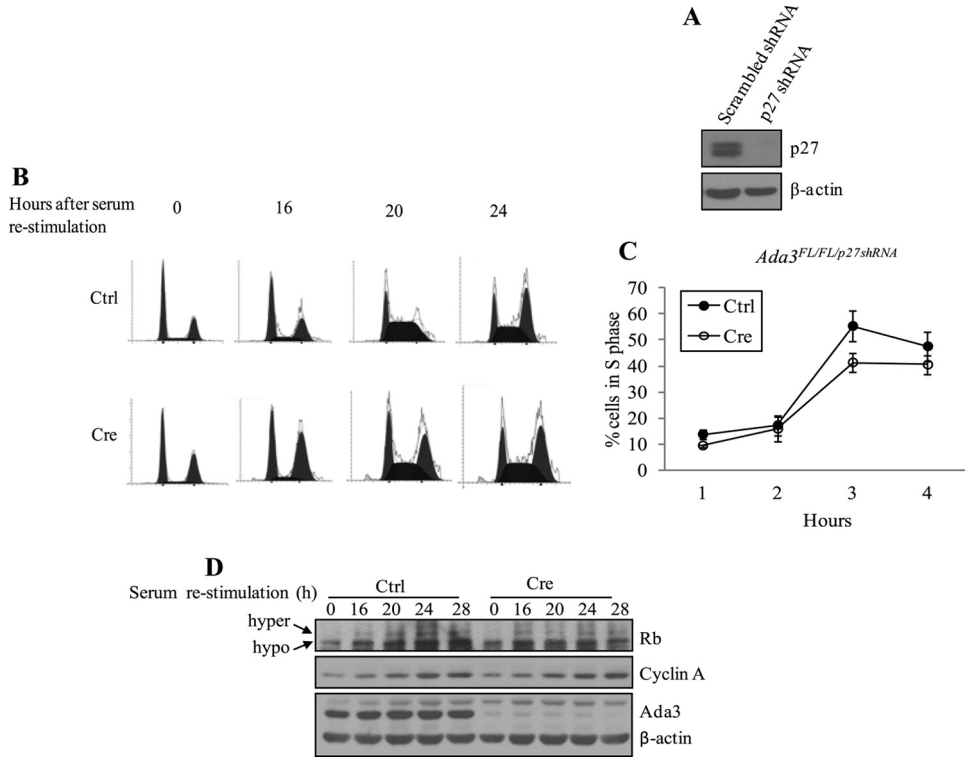


FIGURE 5. **p27 depletion partially rescues G_1 to S transition defects seen in *Ada3*-null MEFs.** *A*, *Ada3*$^{FL/FL}$ MEFs were infected with retrovirus-expressing scrambled or p27 shRNA followed by selection for 2 days in puromycin and analyzed by immunoblotting using p27 and β-actin antibodies. *B*, PI staining and FACS analysis of *Ada3*$^{FL/FL}$ MEFs expressing p27 shRNA that were infected with either control (*Ctrl*) or Cre adenoviruses and synchronized as in Fig. 2. *C*, graph derived from three experiments as in *B* showing the proportion of cells entering into S phase at the indicated times after serum restimulation. *Error bars* indicate mean ± S.E. from three independent experiments. *D*, immunoblotting of protein samples from *B* showing rescue of hyperphosphorylated (*hyper*) Rb and cyclin A levels. *hypo*, hypophosphorylated.

Recombinant Ada3 Stabilizes HAT Enzymes and Enhances Their Activity—Ada3 protein has been identified as an important component of protein complexes containing HAT enzymes. Therefore, we subjected samples harvested after 3 days of *Ada3* deletion to immunoblotting with two important HATs such as p300 and PCAF. Indeed, deletion of *Ada3* caused drastic down-regulation of p300 and PCAF in MEFs (Fig. 7*C*). Notably, *Ada3* deletion had no effect on the mRNA levels of p300 and PCAF (data not shown). Thus, the defects in histone acetylation seen in *Ada3*-null MEFs could be attributed to the effect of *Ada3* deletion on stability of important HATs in cells.

In addition to the role of Ada3 in stability of HAT enzymes, we explored whether Ada3 catalyzes the activity of HAT enzymes. Although Ada3 is shown to be important in maintaining stability of HAT complexes, it has not been demonstrated whether Ada3 directly modulates the activity of known HAT enzymes such as p300. Thus, we expressed and purified baculoviral hAda3 and used it in an *in vitro* assay in which HAT activity of p300 histone acetyl transferase enzyme on histone substrates was measured. As seen in Fig. 7*D*, increasing amounts of Ada3 resulted in increased acetylation of histone H1 and histone H3 by p300, suggesting that Ada3 plays an important role in enhancing the HAT activity of p300. To further explore the role of Ada3 in histone acetylation, we used only histone H3 as a substrate and observed an Ada3 dose-dependent increase in acetylation of histone H3 by p300 (Fig. 7*E*). Thus, Ada3 manifests its effect on histone acetylation by maintaining the integrity of various HAT complexes and by enhancing the catalytic activity of HATs.

Deletion of Ada3 Leads to Global Gene Expression Changes—Given the links between Ada3 and transcriptional activation, we used control and *Ada3*-deleted cells to perform microarray analyses. As expected, the expression of multiple genes was altered; 539 genes were down-regulated and 928 genes were up-regulated ≥ 1.5-fold upon Ada3 deletion (supplemental Table S1). Validation of some of the deregulated genes from microarray by real-time PCR showed good co-relation with the microarray data (supplemental Fig. S4). Ingenuity pathway analyses showed that most of the genes affected were involved in controlling cell growth, proliferation, and cell death (supplemental Table S2, top biological functions affected; cell growth and proliferation (386 genes) and cell death (359 genes)). The top network affected was the RNA post-transcriptional modification and cellular assembly and organization network, whereas the cell cycle, endocrine system development and function, and cancer network was the third most affected network (supplemental Fig. S5). Notably, c-*myc* and *Skp2* genes that we described above were down-regulated 1.4- and 1.43-fold, respectively. This is lower than what we observed by real-time PCR and could be attributed to the fact that microarray data were performed on asynchronous populations, whereas the real-time PCR data were performed on synchronous cells (Fig. 6, *A* and *C*). Interestingly, many of the genes present in cell growth and proliferation set were those involved in controlling cell division as well as some involved in DNA replication (Table 2).

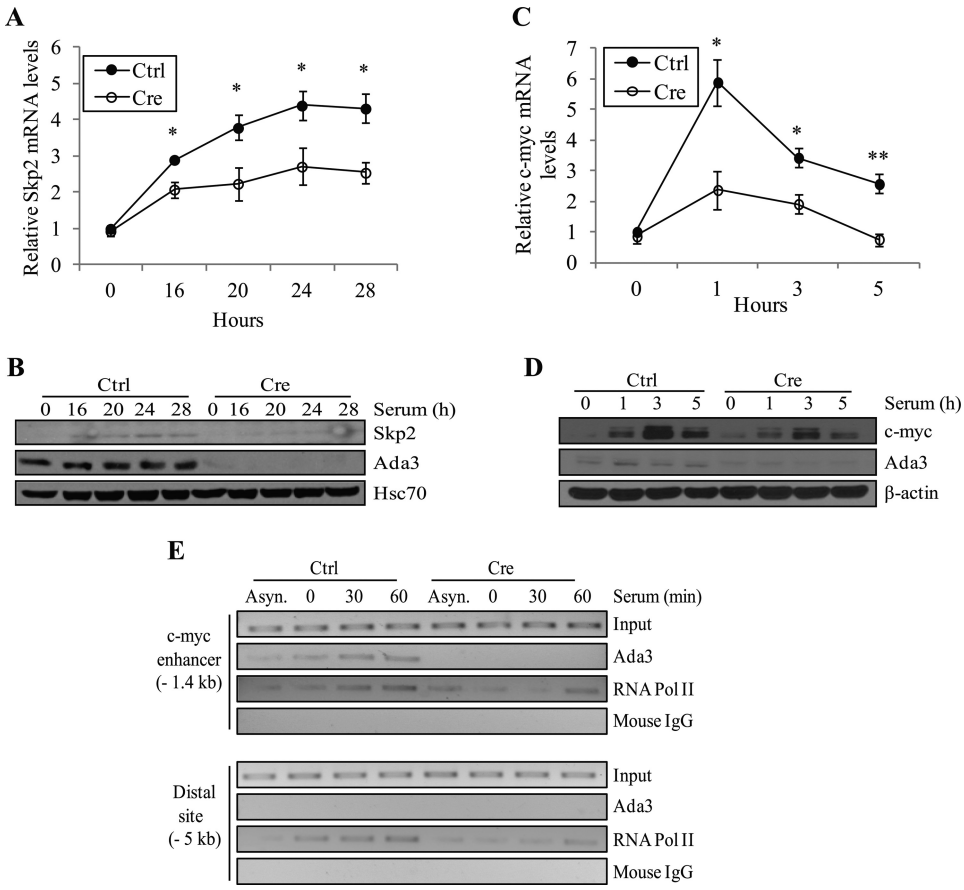


FIGURE 6. **Deletion of *Ada3* from MEFs leads to reduced mRNA and protein levels of Skp2 and c-Myc.** *A*, analysis of Skp2 mRNA levels by real-time RT-PCR from cells as treated in Fig. 2. Signals were normalized to β-actin levels and plotted relative to the level of Skp2 mRNA in starved control cells. *Error bars* represent mean ± S.E. from three independent experiments (*, $p = 0.015$, 0.036, 0.043, and 0.032 for 16, 20, 24, and 28 h, respectively by two-tailed Student's *t* test). *B*, immunoblots showing Skp2 protein levels in cells treated as in *A*. *C*, analysis of c-Myc mRNA levels by real-time RT-PCR from cells as treated in Fig. 5. Signals were normalized to β-actin levels and plotted as in *A*. *Error bars* show mean ± S.E. from three independent experiments. *D*, immunoblots showing c-Myc protein levels in cells treated as in *C* (*, $p = 0.023$ and 0.027 for 1 and 3 h, respectively; **, $p = 0.008$ by two-tailed Student's *t* test). *E*, occupancy of Ada3 on the c-*myc* enhancer. Chromatin fragments from control (*Ctrl*) and Cre *Ada3*$^{FL/FL}$ MEFs cells were immunoprecipitated with anti-Ada3 antibody. Chromatin fragments were prepared from Asynchronous (*Asyn.*) cells as well as from cells synchronized with 0.1% serum containing DMEM for 72 h (*lane 0*) and stimulated with serum with indicated time points. The immunoprecipitated DNA was analyzed by PCR, using c-Myc enhancer-specific primers. Primers amplifying a region that is 5 kb upstream of the c-Myc enhancer were used as a negative control. *RNA Pol II*, RNA polymerase II.

Ada3 Deletion Leads to Defects in Cell Division and Accumulation of Abnormal Nuclei—Based on our microarray analyses where several mitotic genes were affected upon deletion of *Ada3* and a recent study showing the role of Ada3 in mitosis upon shRNA deletion (14), we examined the effect of Ada3 deletion on mitotic phase of cell cycle. These analyses showed that Cre-mediated *Ada3* deletion led to increased accumulation of cells with abnormal nuclei when compared with control MEFs. *Ada3*-deficient MEFs showed various nuclear abnormalities such as fragmentation, lobulation, and multinucleation (Fig. 8*A*). When compared with 13.08 ± 2.39 S.E. % control MEFs, 83.41 ± 3.45 S.E. % of *Ada3*-deficient MEFs showed abnormal nuclei (Fig. 8*B*). Live imaging of cells for 24 h showed that the majority of *Ada3*-deleted cells failed to divide normally. Some of the cells snapped back while attempting to undergo cytokinesis, leading to the formation of binucleated cells, whereas other cells that had normal nucleus before mitosis showed fragmented nuclei afterward and were unable to divide. In other cases, cell division resulted in the formation of anucleated daughter cells (Representative images shown in supplemental Fig. S6). Taken together, these results demonstrate an indispensable role of Ada3 in normal cell cycle progression. The cell division defect results reported here corroborate with an earlier published study showing similar defects upon shRNA knockdown of Ada3 (14). Mitotic defects observed in their study were attributed to acetylation of a non-histone substrate cyclin A, and no changes in histone acetylation upon knockdown of Ada3 were reported. In contrast, we observed a dramatic change in global histone acetylation and expression of various genes involved in mitosis. Although at present we cannot explain this discrepancy, the differences in the results may be partly attributable to the use of different cellular systems and differences in approaches followed such as shRNA or Cre-mediated to delete *Ada3*.

Deletion of Ada3 Leads to Delay in G_2/M to G_1 Progression—As deletion of *Ada3* in MEFs led to defects in cell division, we reasoned that the disruption of Ada3 should exert an effect on G_2/M to G_1 transition. To examine this effect, we synchronized control- and Cre-adenovirus-infected *Ada3*$^{FL/FL}$ MEFs at

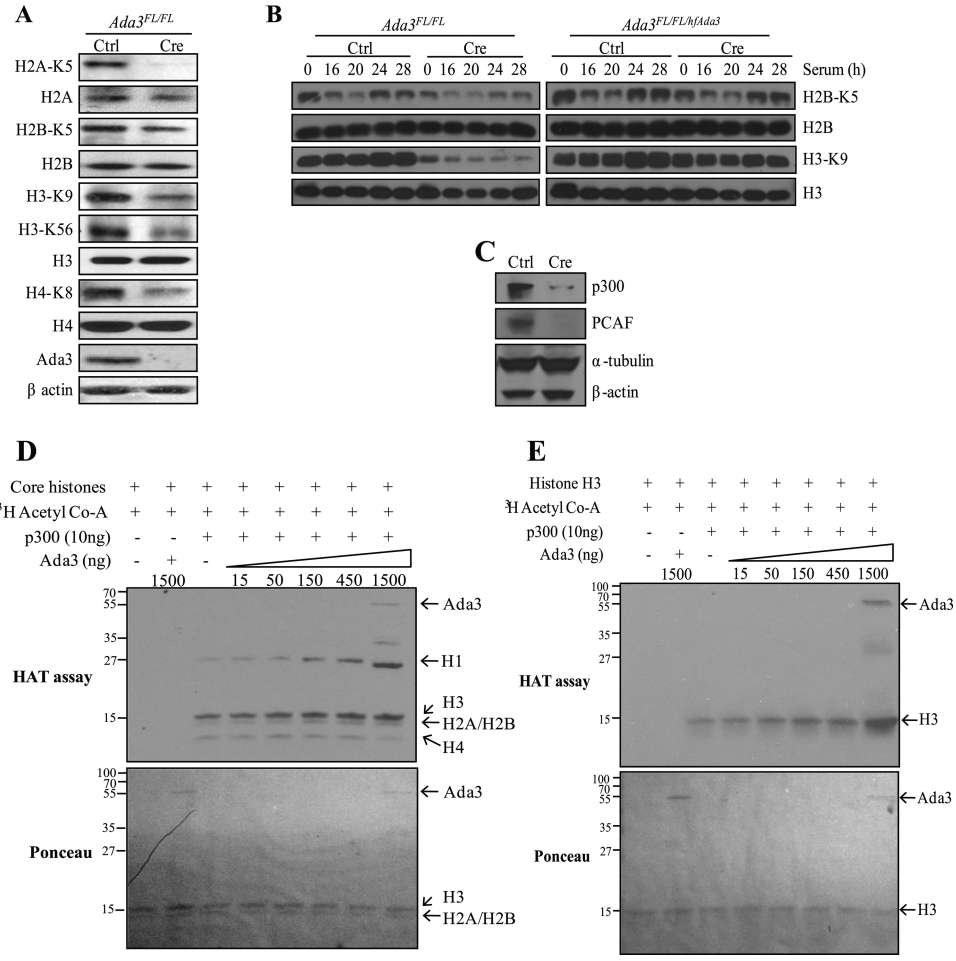


FIGURE 7. ***Ada3* deletion abrogates histone acetylation by destabilizing various HATs.** *A–C*, Western blotting analysis of lysates from asynchronous (*A* and *C*) or serum-restimulated (*B*) $Ada3^{FL/FL}$ or $Ada3^{FL/FL/fhAda3}$ MEFs infected with control (*Ctrl*) or Cre adenoviruses using the indicated antibodies. *D* and *E*, Ada3 enhances p300 HAT activity. *In vitro* HAT assay using purified recombinant human Ada3 and core histones (*D*) or histone H3 alone (*E*) along with their respective Ponceau blots to indicate equal loading is shown.

G_2/M checkpoint by treating them with nocodazole and released them from synchrony followed by cell cycle analysis using flow cytometry (Fig. 8*C*). Nocodazole-synchronized *Ada3*-deleted MEFs showed a lower percentage of cells in G_2/M phase (61%) at the 0-h time point when compared with control MEFs (80%) (Fig. 5*C*). On the contrary, we observed a higher percentage (20%) of *Ada3*-deleted MEFs in G_1 phase when compared with control MEFs (7%) after synchronization. We speculate that *Ada3*-deficient MEFs that are exhibiting a delay in G_1 to S transition were unable to get completely synchronized at G_2/M checkpoint as these cells are potentially moving slowly through the G_1 to S transition and require a prolonged treatment with nocodazole to show a complete synchronization as seen in control MEFs. When we compared the percentage of cells moving into G_1 phase on release from nocodazole treatment in both *Ada3*-deficient and control MEFs, a significant impairment in G_2/M to G_1 transition in *Ada3*-deleted MEFs was observed (Fig. 8*D*). Taken together, these results demonstrate a critical role of Ada3 in both G_1 to S transition as well as G_2/M to G_1 transition in MEFs, indicating that the cell proliferation defect observed in *Ada3*-deficient MEFs is due to a combined defect in G_1 to S as well as G_2/M to G_1 transition.

DISCUSSION

Regulated cell cycle entry and progression are essential for precise developmental programs as well as to maintain organ homeostasis in adult animals. Although the basic components of cell cycle have been largely defined, regulatory control mechanisms that ensure orderly proliferative responses to physiological cues and whose aberrations underlie the vast instances of altered proliferation in cancer continue to be elucidated. We previously identified the ADA complex component Ada3 as a human papillomavirus E6 oncoprotein partner as well as a coactivator of cell cycle checkpoint regulator and tumor suppressor p53 (15, 20). Several *in vitro* studies have shown that Ada3 is an essentially universal component of a multitude of HAT-based transcriptional regulatory complexes (8, 9), and it has become essential to define its physiological roles using *in vivo* animal models.

Here, we demonstrate that Ada3 is essential for embryonic development in mice and that *Ada3*-null embryos undergo very early lethality. As an essential component of the transcriptional coactivator complexes that include HATs and promote histone acetylation of key gene targets, Ada3 is known to be essential for growth in yeast (16) as well as in model metazoan organisms

TABLE 2

List of deregulated genes involved in cell division and DNA replication

Genes down-regulated at least 1.5-fold upon loss of Ada3 as obtained from microarray analyses. The genes were classified based upon gene ontology biological processes.

Gene symbol	Gene title	-Fold down-regulated
Genes involved in cell division		
Kifc1	Kinesin family member C1, similar to Kifc1 protein	2.0
Nfkbil1	Nuclear factor of [kappa[light polypeptide gene enhancer in B-cells inhibitor-like 1	2.0
Fbxo5	F-box protein 5	1.8
Cenpf	Centromere protein F	1.8
Cdc6	Cell division cycle 6 homolog (*Saccharomyces cerevisiae*)	1.7
Kntc1	Kinetochore-associated 1	1.7
Baz1b	Bromodomain adjacent to zinc finger domain, 1B	1.6
Mlf1ip	Myeloid leukemia factor 1 interacting protein	1.6
Myh10	Myosin, heavy polypeptide 10, non-muscle	1.6
Kif11	Kinesin family member 11	1.6
Ccna2	Cyclin A2	1.6
Smc2	Structural maintenance of chromosomes 2	1.6
Plk1	Polo-like kinase 1 (*Drosophila*)	1.5
Bub1b	Budding uninhibited by benzimidazoles 1 homolog, β (*S. cerevisiae*)	1.5
Aspm	asp (abnormal spindle)-like, microcephaly-associated (*Drosophila*)	1.5
Anln	Anillin, actin-binding protein	1.5
Zwilch	Zwilch, kinetochore-associated, homolog (*Drosophila*)	1.5
Mki67	Antigen identified by monoclonal antibody Ki 67	1.5
Mad2l1	MAD2 mitotic arrest deficient-like 1 (yeast)	1.5
Smc4	Structural maintenance of chromosomes 4	1.5
Cdca8	Cell division cycle-associated 8	1.5
Kif20b	Kinesin family member 20B	1.5
Hells	Helicase, lymphoid-specific	1.5
Ccnb1	Cyclin B1	1.5
Cdca3	Cell division cycle-associated 3	1.5
Nuf2	NUF2, NDC80 kinetochore complex component, homolog (*S. cerevisiae*)	1.5
Ndc80	NDC80 homolog, kinetochore complex component (*S. cerevisiae*)	1.5
Birc5	Baculoviral IAP repeat-containing 5	1.5
Bub1	Budding uninhibited by benzimidazoles 1 homolog (*S. cerevisiae*)	1.5
Suv39h2	Suppressor of variegation 3–9 homolog 2 (*Drosophila*)	1.5
Aurkb	Aurora kinase B	1.5
Wee1	WEE 1 homolog 1 (*Schizosaccharomyces pombe*)	1.5
Genes involved in DNA replication		
Kitl	Kit ligand	1.9
Prim1	DNA primase, p49 subunit	1.7
Mcm7	Minichromosome maintenance-deficient 7 (*S. cerevisiae*)	1.7
Ccne2	Cyclin E2	1.7
Pola1	Polymerase (DNA directed), alpha 1	1.7
Dtl	Denticleless homolog (*Drosophila*)	1.7
Cdc6	Cell division cycle 6 homolog (*S. cerevisiae*)	1.7
Chtf18	CTF18, chromosome transmission fidelity factor 18 homolog (*S. cerevisiae*)	1.7
Nfib	nuclear factor I/B	1.6
Prim1	DNA primase, p49 subunit	1.6
Orc1l	Origin recognition complex, subunit 1-like (*S. cerevisiae*)	1.6
Rrm1	Ribonucleotide reductase M1	1.6
Rpa1	Replication protein A1	1.6
Cdt1	Chromatin licensing and DNA replication factor 1	1.6
Gins2	GINS complex subunit 2 (Psf2 homolog)	1.5
Rbbp4	Retinoblastoma-binding protein 4	1.5
Chaf1b	Chromatin assembly factor 1, subunit B (p60)	1.5
Tk1	Thymidine kinase 1	1.5

such as *Drosophila* where Ada3 deficiency is associated with arrest in early development (36). However, this study is the first direct demonstration of an essential role of Ada3 in mammalian embryonic development. Notably, the embryonic developmental block imposed by *Ada3* deletion occurs very early, resulting in arrest of development at the blastocyst stage, the stage of embryonic development at which extensive cell proliferation occurs (37). Notably, studies that employed gene knockouts of subunits of several chromatin-modifying complexes, including *Gcn5*, *Trrap*, *Ep300*, *CBP*, *Hdac3*, or *Atac2*, also lead to early embryonic lethality (34, 38–42), consistent with an essential role of chromatin modification machinery in mammalian growth and development. However, except for *Trrap* knockout, which produces lethality at the blastocyst stage (42), knockouts of other genes produce embryonic developmental arrest at much later stages: for example, *Gcn5* (E9.5–E11.5), *Ep300* (E9.5–E10.5), and *Atac2* (E11.5) in comparison with E3.5 block observed in *Ada3*-null mice. The relatively early developmental arrest of *Ada3*-null mice when compared with other regulators could reflect the role of Ada3 as a component of multiple chromatin-remodeling complexes (see Introduction and below). The distinct times of arrest seen with *Gcn5*-null and *Ada3*-null embryos are somewhat surprising and suggest the possibility that Ada3 may mediate early developmental roles through complexes in which Gcn5 is not a critical component or is functionally redundant with other HATs. Consistent with this hypothesis, we observed that *Ada3*-deleted cells exhibit defects in multiple histone acetylations and show decrease in the levels of PCAF and p300 proteins.

We used the conditional deletion feature of the mouse model to assess the critical functional roles of Ada3 by utilizing Cre-dependent gene deletion in MEFs from $Ada3^{FL/FL}$ mice. This system provided a clear evidence that Ada3 plays an essential

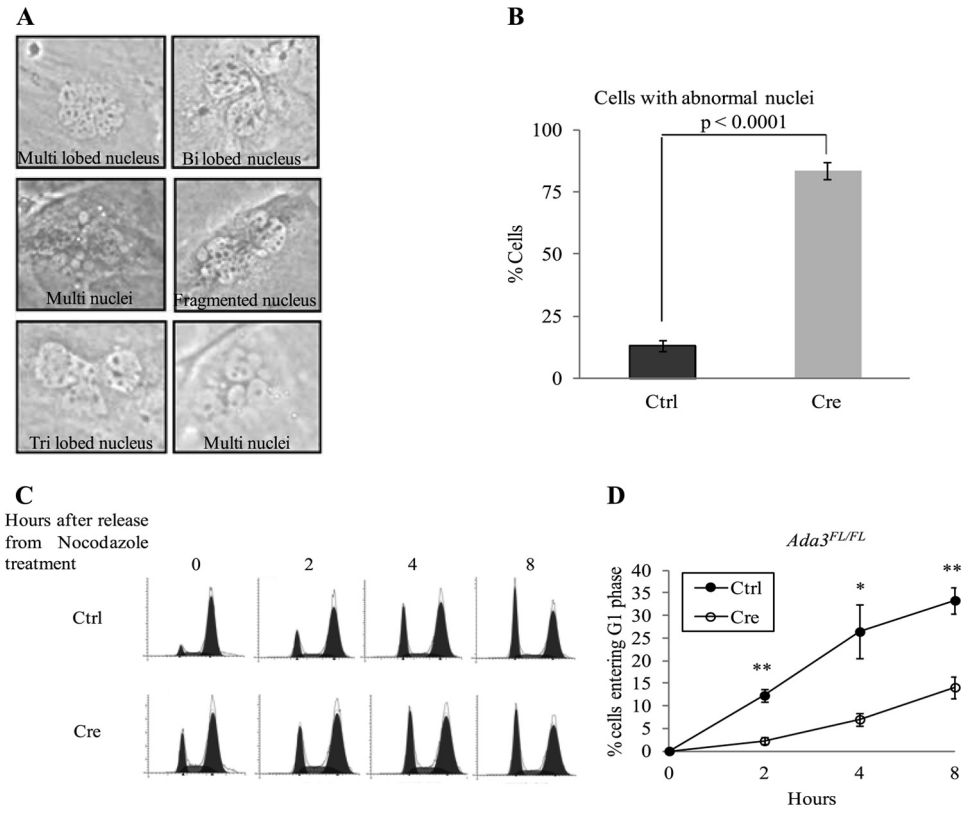


FIGURE 8. **Abnormal cell division and delayed G_2/M to G_1 transition in *Ada3*-deleted cells.** *A*, images of *Ada3*$^{FL/FL}$ cells after 5 days of infection with Cre adenovirus showing abnormal (fragmented, lobulated, or multi) nuclei. *B*, quantification of abnormal nuclei from cells infected with control (*Ctrl*) or Cre adenovirus; 5 days after infection, cells were fixed and stained with Giemsa stain and scored for abnormal nuclei (at least 100 cells from each group were counted). *Error bars* show mean ± S.E. from three independent experiments. *C*, control- and Cre adenovirus-infected MEFs were treated for 20 h with nocodazole and were harvested at the indicated time points after release, stained with PI, and subjected to FACS analysis. *D*, graph showing the percentage of cells entering G_1 phase after release from nocodazole treatment at various time points from experiments as in *C*. *Error bars* are mean ± S.E. from three independent experiments (*, $p = 0.034$; **, $p = 0.0038$ and 0.007 for 4 and 8 h, respectively, by two tailed Student's *t* test).

role in cell proliferation by promoting G_1 to S as well as G_2/M to G_1 cell cycle progression. Furthermore, the proliferative arrest imposed by conditional deletion of *Ada3* was reversed by ectopic expression of human Ada3, indicating that the loss of Ada3 itself, rather than alteration of any neighboring gene product, was responsible for the observed cell cycle phenotype.

Cell cycle progression is a tightly regulated process and is dependent on sequential and stringently controlled, concerted activation of Cdks and their inhibition by Cdk inhibitors. The novel cell cycle regulatory pathway downstream of Ada3 was suggested by our initial analyses of alterations in the levels of core components of mammalian cell cycle machinery. These analyses revealed a dramatic reduction in the key propeller of G_1/S phase transition, hypophosphorylated Rb when *Ada3* was deleted. Association of this defect with reduced Cdk2 activity without a reduction in Cdk2 levels suggested the role of elevated p27, which we established directly by demonstrating that shRNA knockdown of p27 substantially alleviated the G_1/S block imposed by Ada3 deficiency. Further biochemical connections were suggested by recent findings that STAGA complex, which includes Ada3 as a component, enhances c-*myc* transcription (32, 33). Because c-Myc is shown to regulate the transcription of *Skp2*, an essential component of the SCF(Skp2) cell cycle-associated E3 ligase that regulates p27 levels, we sought and established evidence that cell cycle-associated Myc transcription is Ada3-dependent and that Ada3 is required for *Skp2* transcription (which is a downstream target of Myc) and p27 stability (regulated by SCF(Skp2)). We provided direct evidence for key elements of this model, including ChIP analyses that demonstrated the cell cycle-associated early recruitment of Ada3 to c-*myc* enhancer elements. This result is consistent with independent findings from two groups that STAGA complex is recruited to c-Myc enhancer and regulates c-*myc* transcription (32, 33). In addition to control of c-*myc* gene transcription by Ada3-containing STAGA complex, studies have shown that STAGA associates with c-Myc on c-Myc target gene promoters and is required for efficient transcription activation by c-Myc (43, 44). This provides an additional mechanism by which Ada3 could control c-Myc-driven target genes that regulate cell proliferation. Thus, Ada3 might be involved in controlling both c-*myc* transcription as well as c-Myc function. Consistent with our observations, it is noteworthy that c-*myc* knock-out mice are embryonic lethal (45). Defective regulation of c-Myc transcription by Ada3-containing (STAGA or other) complexes might contribute to the early embryonic lethality seen in *Ada3*-null mice; further analyses of Myc-dependent pathways upon germline or conditional deletion of Ada3 during embryogenesis should help establish whether this is the case.

Although regulation of p27 protein stability by Ada3 through control of c-*myc* transcription forms an important basis for

G_1/S transition defects, we were not able to fully rescue the defect in cell cycle by using p27 shRNA, suggesting the involvement of other cellular pathways. To this end, examining global histone acetylations in *Ada3*-deficient cells revealed dramatic defects in histone acetylation. Because Ada3 forms a core structural component of various different HAT complexes in the cell, the presence of Ada3 is highly essential for structural maintenance and proper functioning of these complexes in cells. Additionally, loss of Ada3 led to substantial depletion of important HATs, p300, and PCAF proteins but not mRNA, which further explains the profound defects in histone acetylation seen upon loss of Ada3. This is consistent with the fact that PCAF and p300 are present in Ada3-containing protein complexes (8–11). These defects in histone acetylation could explain the partial rescue upon knockdown of p27 as histone acetylation has been shown to have an important role in the process of DNA replication (34, 35).

Given the role of Ada3 in regulating global histone acetylation and that histone acetylation is important in transcriptional activation of genes, we performed microarray analysis and showed that several genes were deregulated upon *Ada3* deletion. Analysis of these genes by ingenuity pathway analysis revealed the RNA post-transcriptional modification and cellular assembly and organization network as the top affected network, with the cell cycle, endocrine system development and function, and cancer network as the third most affected. The top network affected in the microarray data is consistent with an earlier study, which showed that Ada3-containing STAGA complex interacts with pre-mRNA splicing machinery, components suggesting a role for this complex in mRNA splicing (46). Importantly, the top biological functions affected upon deletion of *Ada3* included those involved in cell growth and proliferation with 386 deregulated genes involved in this process. Thus, our microarray data confirmed a role of Ada3 in cell cycle progression. Additionally, some of the top physiological functions affected upon deletion of Ada3 were those involving tissue development and organismal survival (supplemental Table S2), which could be linked to the early embryonic lethality observed upon knock-out of *Ada3* in mouse.

Notably, many of the genes that were involved in regulating cell growth and proliferation were those involved in mitosis and some that were involved in DNA replication. This led us to examine cell division upon deletion of *Ada3*. Consistent with the microarray data, we observed massive nuclear abnormalities, cell division defects, and delay in G_2/M to G_1 phase progression upon deletion of *Ada3*. Our observed phenomenon of cell division defects upon deletion of *Ada3* is consistent with a recently published study (14). The authors showed that ATAC HAT complex is specifically involved in regulating mitosis and that shRNA-mediated knockdown of Ada3 or Ada2a led to defects in cell division, which were attributed to stabilization of cyclin A upon disruption of ATAC complex. Although we did not observe an increase in cyclin A levels (in fact the converse) in our system, we did observe a similar effect on nuclear abnormalities and a clear defect in mitosis. Furthermore, the authors did not observe any changes in histone acetylation defects upon depletion of Ada3, which is not consistent with our results. Of note, Ada2a is a component of only ATAC complex; however,

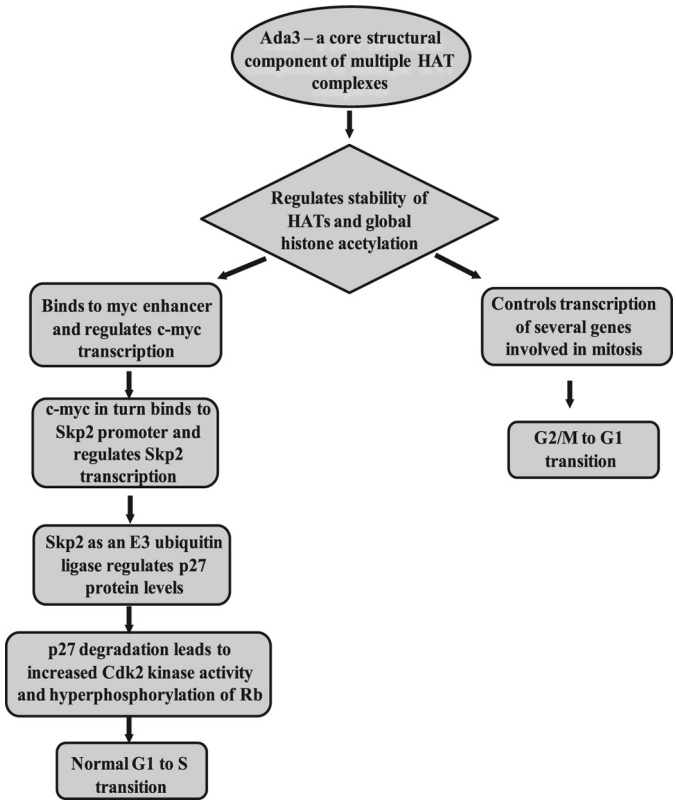


FIGURE 9. **Proposed model for the role of Ada3 in cell cycle progression.** As a core structural component of various HAT complexes, Ada3 maintains the integrity of HAT complexes and thus regulates global histone acetylation. Ada3 regulates G_1 to S transition by controlling transcription of c-*myc* gene, which in turn controls *Skp2* gene expression by binding to its promoter. Skp2 as an E3 ubiquitin ligase causes timely degradation of p27 protein so that cells can enter into S phase by increasing Cdk2 kinase activity, thus inducing hyperphosphorylation of Rb and cell progression from G_1 to S phase of cell cycle. Additionally, through controlling global histone acetylation, Ada3 controls transcription of various genes involved in cell division and is required for cells to undergo normal mitosis and G_2/M to G_1 progression.

Ada3 has been shown to be a core component of a number of HAT complexes. The authors used depletion of Ada3 as an indication of disruption of only ATAC complex; however, deletion of Ada3 would affect several HAT complexes and not just ATAC complex. Thus, deletion of *Ada3* would cause disruption of several HAT complexes that function in different phases of the cell cycle leading to defects in various phases of the cell cycle. Based on these findings, we propose the following working model of Ada3 regulation of cell cycle progression. As part of a chromatin-remodeling complex, likely the STAGA complex, Ada3 is recruited to and modifies the c-*myc* transcriptional regulatory elements to enhance *Skp2* transcription. This leads to destabilization of p27 by the SCF(Skp2) E3 ligase, resulting in increased Cdk2 activity and Rb phosphorylation to promote G_1/S progression. Additionally, Ada3, by regulating the number of genes involved in mitosis, regulates cell division. Lastly, Ada3 as part of ATAC and STAGA complex regulates transcription of various genes by recruiting HATs and acetylating histones. Combination of these functions led to severe cell cycle defect and embryonic lethality upon *Ada3* deletion (Fig. 9). Finally, although our studies here have focused on the role of Ada3 in cell cycle progression, future studies using cell type- or stage-specific condi-

tional deletion of *Ada3* in mouse to assess its role in functions other than transcriptional activation, including optimal transcription elongation, mRNA export, and nucleotide excision repair, need to be explored (8, 46, 47).

In conclusion, we demonstrate that the evolutionarily conserved Ada3 protein as an essential component of HAT complex plays an important role in embryogenesis and cell division. Thus, our studies identify Ada3 as a novel component of the physiological regulation of mammalian cell cycle progression and set the stage for future studies to assess the role of Ada3 in cell cycle progression during *in vivo* physiological and pathological settings. Use of $Ada3^{FL/FL}$ mice should facilitate these analyses to functionally dissect the *in vivo* roles of Ada3.

Acknowledgments—We acknowledge technical assistance from Valerie Tran and Poonam Joshi as well as assistance with time-lapse microscopy from Tom Dao. The University of Nebraska Medical Center (UNMC) DNA Microarray Core facility is supported by grants from the National Center for Research Resources (5P20RR016469) and the NIGMS (8P20GM103427), a component of the National Institutes of Health.

REFERENCES

1. Schafer, K. A. (1998) The cell cycle: a review. *Vet. Pathol* **35,** 461–478
2. Li, B., Carey, M., and Workman, J. L. (2007) The role of chromatin during transcription. *Cell* **128,** 707–719
3. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389,** 251–260
4. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128,** 693–705
5. Strahl, B. D., and Allis, C. D. (2000) The language of covalent histone modifications. *Nature* **403,** 41–45
6. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) Histone acetyltransferases. *Annu. Rev. Biochem.* **70,** 81–120
7. Carrozza, M. J., Utley, R. T., Workman, J. L., and Côté, J. (2003) The diverse functions of histone acetyltransferase complexes. *Trends Genet.* **19,** 321–329
8. Lee, K. K., and Workman, J. L. (2007) Histone acetyltransferase complexes: one size doesn't fit all. *Nat. Rev. Mol. Cell Biol.* **8,** 284–295
9. Nagy, Z., and Tora, L. (2007) Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene* **26,** 5341–5357
10. Wang, T., Kobayashi, T., Takimoto, R., Denes, A. E., Snyder, E. L., el-Deiry, W. S., and Brachmann, R. K. (2001) hADA3 is required for p53 activity. *EMBO J.* **20,** 6404–6413
11. Germaniuk-Kurowska, A., Nag, A., Zhao, X., Dimri, M., Band, H., and Band, V. (2007) Ada3 requirement for HAT recruitment to estrogen receptors and estrogen-dependent breast cancer cell proliferation. *Cancer Res.* **67,** 11789–11797
12. Vernarecci, S., Ornaghi, P., Bâgu, A., Cundari, E., Ballario, P., and Filetici, P. (2008) Gcn5p plays an important role in centromere kinetochore function in budding yeast. *Mol. Cell. Biol.* **28,** 988–996
13. Paolinelli, R., Mendoza-Maldonado, R., Cereseto, A., and Giacca, M. (2009) Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. *Nat. Struct. Mol. Biol.* **16,** 412–420
14. Orpinell, M., Fournier, M., Riss, A., Nagy, Z., Krebs, A. R., Frontini, M., and Tora, L. (2010) The ATAC acetyl transferase complex controls mitotic progression by targeting non-histone substrates. *EMBO J.* **29,** 2381–2394
15. Kumar, A., Zhao, Y., Meng, G., Zeng, M., Srinivasan, S., Delmolino, L. M., Gao, Q., Dimri, G., Weber, G. F., Wazer, D. E., Band, H., and Band, V. (2002) Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. *Mol. Cell. Biol.* **22,** 5801–5812
16. Piña, B., Berger, S., Marcus, G. A., Silverman, N., Agapite, J., and Guarente, L. (1993) ADA3: a gene, identified by resistance to GAL4-VP16, with properties similar to and different from those of ADA2. *Mol. Cell. Biol.* **13,** 5981–5989
17. Horiuchi, J., Silverman, N., Marcus, G. A., and Guarente, L. (1995) ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex. *Mol. Cell. Biol.* **15,** 1203–1209
18. Saleh, A., Lang, V., Cook, R., and Brandl, C. J. (1997) Identification of native complexes containing the yeast coactivator/repressor proteins NGG1/ADA3 and ADA2. *J. Biol. Chem.* **272,** 5571–5578
19. Eberharter, A., Sterner, D. E., Schieltz, D., Hassan, A., Yates, J. R., 3rd, Berger, S. L., and Workman, J. L. (1999) The ADA complex is a distinct histone acetyltransferase complex in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19,** 6621–6631
20. Nag, A., Germaniuk-Kurowska, A., Dimri, M., Sassack, M. A., Gurumurthy, C. B., Gao, Q., Dimri, G., Band, H., and Band, V. (2007) An essential role of human Ada3 in p53 acetylation. *J. Biol. Chem.* **282,** 8812–8820
21. Zeng, M., Kumar, A., Meng, G., Gao, Q., Dimri, G., Wazer, D., Band, H., and Band, V. (2002) Human papilloma virus 16 E6 oncoprotein inhibits retinoic X receptor-mediated transactivation by targeting human ADA3 coactivator. *J. Biol. Chem.* **277,** 45611–45618
22. Zhao, Y., Lang, G., Ito, S., Bonnet, J., Metzger, E., Sawatsubashi, S., Suzuki, E., Le Guezennec, X., Stunnenberg, H. G., Krasnov, A., Georgieva, S. G., Schüle, R., Takeyama, K., Kato, S., Tora, L., and Devys, D. (2008) A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. *Mol Cell* **29,** 92–101
23. Todaro, G. J., and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17,** 299–313
24. Weinberg, R. A. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81,** 323–330
25. Dyson, N. (1998) The regulation of E2F by pRB family proteins. *Genes Dev.* **12,** 2245–2262
26. Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994) Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* **372,** 570–573
27. Reynisdóttir, I., Polyak, K., Iavarone, A., and Massagué, J. (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. *Genes Dev.* **9,** 1831–1845
28. Sherr, C. J., and Roberts, J. M. (1999) CDK inhibitors: positive and negative regulators of G_1 phase progression. *Genes Dev.* **13,** 1501–1512
29. Besson, A., Gurian-West, M., Chen, X., Kelly-Spratt, K. S., Kemp, C. J., and Roberts, J. M. (2006) A pathway in quiescent cells that controls $p27^{Kip1}$ stability, subcellular localization, and tumor suppression. *Genes Dev.* **20,** 47–64
30. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat. Cell Biol.* **1,** 193–199
31. Bretones, G., Acosta, J. C., Caraballo, J. M., Ferrándiz, N., Gómez-Casares, M. T., Albajar, M., Blanco, R., Ruiz, P., Hung, W. C., Albero, M. P., Perez-Roger, I., and León, J. (2011) SKP2 oncogene is a direct MYC target gene, and MYC down-regulates p27(KIP1) through SKP2 in human leukemia cells. *J. Biol. Chem.* **286,** 9815–9825
32. Chen, J., Luo, Q., Yuan, Y., Huang, X., Cai, W., Li, C., Wei, T., Zhang, L., Yang, M., Liu, Q., Ye, G., Dai, X., and Li, B. (2010) Pygo2 associates with MLL2 histone methyltransferase and GCN5 histone acetyltransferase complexes to augment Wnt target gene expression and breast cancer stem-like cell expansion. *Mol. Cell. Biol.* **30,** 5621–5635
33. Yang, M., Waterman, M. L., and Brachmann, R. K. (2008) hADA2a and hADA3 are required for acetylation, transcriptional activity, and proliferative effects of β-catenin. *Cancer Biol. Ther.* **7,** 120–128
34. Bhaskara, S., Chyla, B. J., Amann, J. M., Knutson, S. K., Cortez, D., Sun, Z. W., and Hiebert, S. W. (2008) Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. *Mol. Cell*

30, 61–72
35. Burgess, R. J., Zhou, H., Han, J., and Zhang, Z. (2010) A role for Gcn5 in replication-coupled nucleosome assembly. *Mol. Cell* **37,** 469–480
36. Grau, B., Popescu, C., Torroja, L., Ortuño-Sahagún, D., Boros, I., and Ferrús, A. (2008) Transcriptional adaptor ADA3 of *Drosophila melanogaster* is required for histone modification, position effect variegation, and transcription. *Mol. Cell. Biol.* **28,** 376–385
37. Ciemerych, M. A., and Sicinski, P. (2005) Cell cycle in mouse development. *Oncogene* **24,** 2877–2898
38. Yao, T. P., Oh, S. P., Fuchs, M., Zhou, N. D., Ch'ng, L. E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., and Eckner, R. (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell* **93,** 361–372
39. Yamauchi, T., Yamauchi, J., Kuwata, T., Tamura, T., Yamashita, T., Bae, N., Westphal, H., Ozato, K., and Nakatani, Y. (2000) Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 11303–11306
40. Kasper, L. H., Fukuyama, T., Biesen, M. A., Boussouar, F., Tong, C., de Pauw, A., Murray, P. J., van Deursen, J. M., and Brindle, P. K. (2006) Conditional knockout mice reveal distinct functions for the global transcriptional coactivators CBP and p300 in T-cell development. *Mol. Cell. Biol.* **26,** 789–809
41. Guelman, S., Kozuka, K., Mao, Y., Pham, V., Solloway, M. J., Wang, J., Wu, J., Lill, J. R., and Zha, J. (2009) The double-histone-acetyltransferase complex ATAC is essential for mammalian development. *Mol. Cell. Biol.* **29,** 1176–1188
42. Herceg, Z., Hulla, W., Gell, D., Cuenin, C., Lleonart, M., Jackson, S., and Wang, Z. Q. (2001) Disruption of Trrap causes early embryonic lethality and defects in cell cycle progression. *Nat. Genet.* **29,** 206–211
43. Liu, X., Tesfai, J., Evrard, Y. A., Dent, S. Y., and Martinez, E. (2003) c-Myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation. *J. Biol. Chem.* **278,** 20405–20412
44. Liu, X., Vorontchikhina, M., Wang, Y. L., Faiola, F., and Martinez, E. (2008) STAGA recruits Mediator to the MYC oncoprotein to stimulate transcription and cell proliferation. *Mol. Cell. Biol.* **28,** 108–121
45. Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993) A null c-*myc* mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev.* **7,** 671–682
46. Martinez, E., Palhan, V. B., Tjernberg, A., Lymar, E. S., Gamper, A. M., Kundu, T. K., Chait, B. T., and Roeder, R. G. (2001) Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors *in vivo*. *Mol. Cell. Biol.* **21,** 6782–6795
47. Torok, M. S., and Grant, P. A. (2004) Histone acetyltransferase proteins contribute to transcriptional processes at multiple levels. *Adv. Protein Chem.* **67,** 181–199

BioArchitecture 1:1, 45-48; January/February 2011; © 2011 Landes Bioscience

ESCRT proteins

Double-edged regulators of cellular signaling

Chun Tu,[1,†] Gulzar Ahmad,[1] Bhopal Mohapatra,[1,2] Sohinee Bhattacharyya,[1,3] Cesar F. Ortega-Cava,[1] Byung Min Chung,[5] Kay-Uwe Wagner,[1] Srikumar M. Raja,[1] Mayumi Naramura,[1] Vimla Band[1,4] and Hamid Band[1,2,*]

[1]Eppley Institute for Research in Cancer and Allied Diseases and UNMC-Eppley Cancer Center; [2]Department of Biochemistry and Molecular Biology; [3]Department of Pathology and Microbiology; [4]Department of Genetics, Cell Biology and Anatomy; University of Nebraska Medical Center; Omaha, NE USA; [5]Department of Biochemistry and Molecular Biology; Bloomberg School of Public Health; Johns Hopkins University; Baltimore, MD USA

Current Address: [†]MD Anderson Cancer Center; University of Texas; Houston, TX USA

ESCRT pathway proteins play a key role in sorting ubiquitinated membrane receptors towards lysosomes providing an important mechanism for attenuating cell surface receptor signaling. However, recent studies point to a positive role of ESCRT proteins in signal transduction in multiple species studied under physiological and pathological conditions. ESCRT components such as Tsg101 and Hrs are overexpressed in human cancers and Tsg101 depletion is detrimental for cell proliferation, survival and transformed phenotype of tumor cells. However, the mechanisms underlying the positive contributions of ESCRT pathway to surface receptor signaling have remained unclear. In a recent study, we showed that Tsg101 and Vps4 are essential for translocation of active Src from endosomes to focal adhesion and invadopodia, thereby revealing a role of ESCRT pathway in promoting Src-mediated migration and invasion. We discuss the implications of these and other recent studies which together suggest a role for the ESCRT pathway in recycling of endocytic cargo proteins, aside from its role in lysosomal targeting, potentially explaining the positive roles of ESCRT proteins in signal transduction.

Key words: endocytotic trafficking, recycling, ESCRT proteins, Src, Signal transduction, cancer, lysosomal degradation, receptor tyrosine kinase

Submitted: 02/13/11

Accepted: 02/15/11

DOI: 10.4161/bioa.1.1.15173

*Correspondence to: Hamid Band;
Email: hband@unmc.edu

Endocytosis of cell surface receptors is a fundamental cell biological process. Upon their entry into early endosomal compartments, internalized receptors undergo a sorting process that determines their alternate itineraries. For example, activated receptor tyrosine kinases (RTKs) internalized upon stimulation by growth factors can be alternatively targeted to the lysosome where they are degraded or recycled back to the cell surface through the recycling endocytic pathway for further rounds of growth factor binding and signaling. Thus, the endocytic route of internalized receptors can control the duration and strength of activation signals. Therefore, mechanisms that control the alternative lysosomal versus recycling fates of internalized receptors have received substantial attention in recent years. Ubiquitination has emerged as a key mechanism to target cell surface receptors for lysosomal degradation. In a mechanism that is conserved from yeast to mammals, the sorting of ubiquitinated receptors is orchestrated by a series of protein complexes collectively known as Endosomal Sorting Complexes Required for Transport or ESCRT proteins (reviewed in ref. 1). The components and the arrangement of the currently described four ESCRT complexes, ESCRT-0, -I, -II and -III, as well as their associated proteins are highly conserved. The heterotetrameric ESCRT-I complex includes the proteins Vacuolar sorting protein (Vps) 23 (known as Tumor suppressor gene 101 or Tsg101 in mammals), Vps28, Vps37 and Multivesicular body protein (MVB) 12. Tsg101 plays a key role in ESCRT-I function as it can bind to ubiquitinated receptors and mediates interactions with other

ESCRT complexes via its ubiquitin-conjugating enzyme E2 variant (UEV) domain.

Since the lysosomal targeting of activated cell surface receptors is now a well accepted mechanism of signal attenuation,+ much attention has naturally focused on the negative regulatory roles of the ESCRT pathway especially in the context of RTK signaling in mammalian cell systems. Yet, a large number of studies, especially those done in organismic contexts, have revealed essential positive roles of ECSRT proteins. Analyses of Tsg101 provide the clearest example of these diametrically opposite functional roles.

While initial identification of Tsg101 as a transformation suppressor apparently mutated in breast cancers was consistent with the negative regulatory role of ESCRT complexes in receptor signaling, later studies did not confirm these findings.[2] Remarkably, several independent studies using genetic ablation approaches showed that Tsg101 was required for mouse embryonic development and viability of adult tissues and cells.[3-5] A similar essential role of Tsg101 homologs and other ESCRT components in embryonic development is shown by studies in model organisms such as *C. elegans* and Drosophila. More recent work has shown that Tsg101 is overexpressed rather than lost in a subset of breast,[5] thyroid,[6] ovarian[7] and colon[8] cancers. Furthermore, depletion of Tsg101 was found to impair tumorigenicity of several cancer cell lines.[9,10]

ESCRT proteins are increasing recognized as versatile proteins functioning in diverse membrane abscission processes. Extensive studies for example have shown the requirement of ESCRT functions for retroviral budding as well as orchestrating several cellular processes such as autophagy and cytokinesis (reviewed by ref. 1). These findings have painted a more complex picture of the roles played by Tsg101 as well as other ESCRT proteins.

While the seemingly positive roles in signal transduction have been defined most clearly for Tsg101, other ESCRT proteins are likely to behave in a similar manner. For example, the ESCRT-0 component Hepatocyte growth factor receptor substrate (Hrs) was found to be essential for cell proliferation, anchorage-independent growth, tumorigenesis and metastatic potential of HeLa cells and mouse fibroblasts in vitro and in vivo.[11] In addition, overexpression of Hrs was found to be associated with advanced malignancy and poor prognosis in human cancers.[11] Interestingly, both positive and negative[12,13] roles of Hrs in the signaling have been reported in Drosophila and mammalian cells, suggesting that the exact role of Hrs could be cell type- and context-dependent.

The ability of Tsg101, and perhaps other ESCRT proteins, to serve positive as well as negative roles in cellular homeostasis necessitate a better understanding of their complex biochemical and cellular functions. In this regard, Tsg101 and other ESCRT proteins share some features of the so-called "two-faced" proteins that are being increasingly recognized in many fields and can regulate biological functions in seemingly opposite ways under different physiological scenarios. As a notable example of this expanding group of proteins, TGFβ can exhibit an oncogenic role in one context but an exactly opposite tumor suppressor role in a different context.

The question of how ESCRT proteins such as Tsg101 and Hrs serve positive roles in cellular signaling has remained largely unanswered, until recently. In one potential scenario, the seemingly opposite functions of ESCRT proteins could relate to their distinct complexes or subcellular pools, although there is no clear evidence for this idea at this time. Alternatively, distinct ESCRT-mediated functions may reflect their diverse endosomal sorting roles in the context of a broad range of molecular targets that have vastly different roles in cellular homeostasis. For example, ESCRT pathway has been shown to promote lysosomal proteolysis of anti-proliferative proteins such as E-cadherin[11] and GSK-3,[14] which can translate into positive cellular roles of the ESCRT pathway.

Emerging evidence that ESCRT proteins promote sorting of endosomal cargo proteins to destinations other than lysosomes could also underlie their positive roles in cellular signaling. Recently, we demonstrated that Tsg101 and Vps4, two well-studied ESCRT proteins, are required for the translocation of active c-Src tyrosine kinase (Src) from late endosomal/lysosomal compartment to focal adhesions, where Src functions to promote cell motility and to activate downstream effectors such as FAK and STAT3.[15] Additional findings (our unpublished observations) indicate that Tsg101 is also critically required for the formation of Src-dependent structure called invadopodia, membrane protrusions that are known to mediate matrix invasion by highly invasive tumor cells (reviewed in ref. 16). Thus, Tsg101 (and ESCRT complexes) appears to be essential for Src activity and function, by ensuring the dynamic trafficking of Src to its sites of function at focal adhesions and invadopodia.

These findings have several important implications. First, this study revealed a novel connection between ESCRT pathway and the Src tyrosine kinase, the latter a participant in a broad range of cellular responses such as survival, proliferation and migration in normal cells as well as during oncogenesis. Src activity is frequently elevated in a number of human cancers, cooperating with other oncoproteins to confer more invasive characteristics to cancer cells. These findings may explain in part why Tsg101 plays a positive role in cellular signal transduction and why its overexpression rather than reduced expression is common in highly malignant cancers. Obviously, additional studies in other experimental settings will be necessary to validate the functional interaction we have shown between Src and ESCRT pathway, and to test the potential role of this new interaction in oncogenesis. Should the latter be validated, ESCRT pathway could emerge as a potential target to develop new anticancer therapeutic agents.

Secondly, the demonstration that Tsg101 and Vps4 are required for the trafficking of active Src out of late endosomes instead of targeting it for degradation expands the functional roles of ESCRT proteins. Other recent data further point to a general involvement of the ESCRT pathway in the recycling of endosomal cargo. Vps4, which is required for dynamic cycling of ESCRT complexes between the endosomal membrane and the cytoplasm,[17] has been shown to be essential for

trafficking of transferrin receptor, mucin-like receptor endolyn[18] and low density lipoprotein receptor (LDLR) between endosomes and other membrane compartments.[19] Even in the case of classical RTKs whose lysosomal sorting is facilitated by ESCRT proteins, further work and reinterpretation of previous findings may be necessary. For instance, previous studies showing an important role of the ESCRT pathway in RTK dynamics utilized the persistence of labeled ligands such as EGF at endosomes as evidence for a selective defect in lysosomal degradation in cells with dysfunctional ESCRT pathway.[20,21] However, if recycling of EGFR was still operational when the ESCRT machinery was rendered defective, one would expect a larger fraction of EGFR at the plasma membrane; however, this has not been documented. Thus, the phenotypes observed in ESCRT-defective cells may be better explained by a combined block of lysosomal degradation and recycling. This idea is supported by our unpublished data as well as a recent study which showed that recycling of EGFR triggered by stimulation with amphiregulin is reduced upon Tsg101 depletion.[22]

Accumulating evidence indicates that other ESCRT complexes are also implicated in recycling or endocytic cargo proteins. Alix, an ESCRT protein interact with Tsg101 and ESCRT III complex, is required for endocytic recycling of specific basolateral cargo in the *C. elegans*.[23] Hrs has been shown to mediate endocytic recycling of G-protein coupled receptors such as β2 adrenergic receptor,[24] protease-activated receptor 2 (PAR2) and calcitonin receptor-like receptor (CLR).[25] Importantly, this role seems not to be limited to mammalian cells or G-protein coupled receptors. In Drosophila, mutations in Hrs and another ESCRT-0 component STAM have been shown to sequester fibroblast growth factor receptor (FGFR) in aberrantly enlarged early endosomes, in contrast to its normal localization at the plasma membrane (PM).[13] Overexpression of ESCRT III complex component CHMP6 has been shown to cause the retention of transferrin receptor at endosomes.[26] Recently, ESCRT-I function was shown to be essential for transport of cargo proteins from early endosomes to the limiting membrane of melanosomes in melanocytes.[27] These data further boost the notion that ESCRT machinery may be equally important for sorting of endosomal cargoes to inner vesicles of the MVB and other endocytic destinations. This notion is consistent with the emerging idea that different sorting domains coexist in a multifunctional endosomal sorting compartment with ESCRT proteins facilitating sorting of cargo towards multiple destinations.[18,21]

Obviously, further investigations are needed to confirm and extend our knowledge regarding the dual roles of ESCRT proteins in endosomal trafficking. How is endosomal recycling being facilitated by ESCRT complexes? Given our findings using Tsg101-null cells,[15] a reconstitution approach should allow structure-function analysis of Tsg101 to address which domains are required for endosomal recycling, thereby pointing to potential mechanisms. What cargos might be sorted for recycling rather than lysosomal delivery, and what might determine the ratio of degradation versus recycling for a given protein that could be sorted to either destination? It is possible that accessory factors and/or post-translational modifications of cargo proteins in part dictate the destination of the sorted cargo. In this regard, while ubiquitin-modified receptors are targeted by ESCRT complexes for delivery into inner vesicles of the MVB for delivery to the lysosome, it is possible that ESCRT proteins may help sort unmodified receptors back to the cell surface. Consistent with such a scenario, ESCRT-0 complex STAM has been shown to interact with deubiquitinases and this interaction has been demonstrated to help avert the degradation fate of EGFR.[28,29] In addition, the expression of SCAMP3, a protein that interacts with Tsg101 and Alix, was shown to favor EGFR recycling at the expense of lysosomal degradation.[30] Studies using ESCRT-null or -depleted cells should help test these ideas further. Perhaps most importantly, it is pertinent to ask to what extent the ESCRT-facilitated transport of proteins from endosomes to non-lysosomal destinations contributes to the positive roles of ESCRTs in signal transduction? Answers to this question could help reconcile some of the more puzzling observations in ESCRT and endocytosis fields especially as it relates to cellular signaling receptors.

Acknowledgements

This work was supported by National Institutes of Health Grants CA105489, CA87986, CA116552 and CA99163 to H.B., CA96844 to V.B., and CA93797 to K.U.W.; Department of Defense Breast Cancer Research Grant W81XWH-07-1-0351 and W81XWH-11-1-0171 to V.B., W81XWH-11-1-0166 to H.B. and W81 XWH-10-1-0740 to M.N.; University of Nebraska Medical Center (UNMC)-Eppley Cancer Center Pilot Grants to M.N. and V.B.; Nebraska Department of Health and Human Services LB-506 grant to S.M.R.; and Nebraska Center for Nanomedicine-Center for Biomedical Research Excellence (NCN-COBRE) seed grant to S.M.R.

References

1. Saksena S, Sun J, Chu T, Emr SD. ESCRTing proteins in the endocytic pathway. Trends Biochem Sci 2007; 32:561-73.
2. Li L, Francke U, Cohen SN. Retraction the TSG101 tumor susceptibility gene is located in chromosome 11 band p15 and is mutated in human breast cancer. Cell 1998; 93:660.
3. Ruland J, Sirard C, Elia A, MacPherson D, Wakeham A, Li L, et al. p53 accumulation, defective cell proliferation and early embryonic lethality in mice lacking Tsg101. Proc Natl Acad Sci USA 2001; 98:1859-64.
4. Wagner KU, Krempler A, Qi Y, Park K, Henry MD, Triplett AA, et al. Tsg101 is essential for cell growth, proliferation and cell survival of embryonic and adult tissues. Mol Cell Biol 2003; 23:150-62.
5. Oh KB, Stanton MJ, West WW, Todd GL, Wagner KU. Tsg101 is upregulated in a subset of invasive human breast cancers and its targeted overexpression in transgenic mice reveals weak oncogenic properties for mammary cancer initiation. Oncogene 2007; 26:5950-9.
6. Liu RT, Huang CC, You HL, Chou FF, Hu CC, Chao FP, et al. Overexpression of tumor susceptibility gene TSG101 in human papillary thyroid carcinomas. Oncogene 2002; 21:4830-7.
7. Young TW, Rosen DG, Mei FC, Li N, Liu J, Wang XF, Cheng X. Upregulation of tumor susceptibility gene 101 conveys poor prognosis through suppression of p21 expression in ovarian cancer. Clin Cancer Res 2007; 13:3848-54.
8. Ma XR, Edmund Sim UH, Pauline B, Patricia L, Rahman J. Overexpression of WNT2 and TSG101 genes in colorectal carcinoma. Trop Biomed 2008; 25:46-57.
9. Zhu G, Gilchrist R, Borley N, Chng HW, Morgan M, Marshall JF, et al. Reduction of TSG101 protein has a negative impact on tumor cell growth. Int J Cancer 2004; 109:541-7.
10. Shen H, Pan Y, Han Z, Hong L, Liu N, Han S, et al. Reversal of multidrug resistance of gastric cancer cells by downregulation of TSG101 with TSG101siRNA. Cancer Biol Ther 2004; 3:561-5.

11. Toyoshima M, Tanaka N, Aoki J, Tanaka Y, Murata K, Kyuuma M, et al. Inhibition of tumor growth and metastasis by depletion of vesicular sorting protein hrs: Its regulatory role on E-cadherin and beta-catenin. Cancer Res 2007; 67:5162-71.
12. Scoles DR, Nguyen VD, Qin Y, Sun CX, Morrison H, Gutmann DH, Pulst SM. Neurofibromatosis 2 (NF2) tumor suppressor schwannomin and its interacting protein HRS regulate STAT signaling. Hum Mol Genet 2002; 11:3179-89.
13. Chanut-Delalande H, Jung AC, Baer MM, Lin L, Payre F, Affolter M. The Hrs/Stam complex acts as a positive and negative regulator of RTK signaling during drosophila development. PLoS One 2010; 5:10245.
14. Taelman VF, Dobrowolski R, Plouhinec JL, Fuentealba LC, Vorwald PP, Gumper I, et al. Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell 2010; 143:1136-48.
15. Tu C, Ortega-Cava CF, Winograd P, Stanton MJ, Reddi AL, Dodge I, et al. Endosomal-sorting complexes required for transport (ESCRT) pathway-dependent endosomal traffic regulates the localization of active src at focal adhesions. Proc Natl Acad Sci USA 2010; 107:16107-12.
16. Buccione R, Caldieri G, Ayala I. Invadopodia: Specialized tumor cell structures for the focal degradation of the extracellular matrix. Cancer Metastasis Rev 2009; 28:137-49.
17. Bishop N, Woodman P. TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes. J Biol Chem 2001; 276:11735-42.
18. Fujita H, Yamanaka M, Imamura K, Tanaka Y, Nara A, Yoshimori T, et al. A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: Class E vps phenotype in mammalian cells. J Cell Sci 2003; 116:401-14.
19. Yoshimori T, Yamagata F, Yamamoto A, Mizushima N, Kabeya Y, Nara A, et al. The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. Mol Biol Cell 2000; 11:747-63.
20. Bishop N, Horman A, Woodman P. Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. J Cell Biol 2002; 157:91-101.
21. Doyotte A, Russell MR, Hopkins CR, Woodman PG. Depletion of TSG101 forms a mammalian "class E" compartment: A multicisternal early endosome with multiple sorting defects. J Cell Sci 2005; 118:3003-17.
22. Baldys A, Raymond JR. Critical role of ESCRT machinery in EGFR recycling. Biochemistry 2009; 48:9321-3.
23. Shi A, Pant S, Balklava Z, Chen CC, Figueroa V, Grant BD. A novel requirement for *C. elegans* Alix/ALX-1 in RME-1-mediated membrane transport. Curr Biol 2007; 17:1913-24.
24. Hanyaloglu AC, McCullagh E, von Zastrow M. Essential role of hrs in a recycling mechanism mediating functional resensitization of cell signaling. EMBO J 2005; 24:2265-83.
25. Hasdemir B, Bunnett NW, Cottrell GS. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) mediates post-endocytic trafficking of protease-activated receptor 2 and calcitonin receptor-like receptor. J Biol Chem 2007; 282:29646-57.
26. Yorikawa C, Shibata H, Waguri S, Hatta K, Horii M, Katoh K, et al. Human CHMP6, a myristoylated ESCRT-III protein, interacts directly with an ESCRT-II component EAP20 and regulates endosomal cargo sorting. Biochem J 2005; 387:17-26.
27. Truschel ST, Simoes S, Setty SR, Harper DC, Tenza D, Thomas PC, et al. ESCRT-I function is required for Tyrp1 transport from early endosomes to the melanosome limiting membrane. Traffic 2009; 10:1318-36.
28. Niendorf S, Oksche A, Kisser A, Lohler J, Prinz M, Schorle H, et al. Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol Cell Biol 2007; 27:5029-39.
29. Berlin I, Schwartz H, Nash PD. Regulation of epidermal growth factor receptor ubiquitination and trafficking by the USP8.STAM complex. J Biol Chem 2010; 285:34909-21.
30. Aoh QL, Castle AM, Hubbard CH, Katsumata O, Castle JD. SCAMP3 negatively regulates epidermal growth factor receptor degradation and promotes receptor recycling. Mol Biol Cell 2009; 20:1816-32.